DETERMINATION OF PLASMA IOHEXOL BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY: EFFECTS OF AN IMPROVED METHOD IN RENAL AND LIVER DISEASES PATIENTS

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ABSTRACT

Plasma iohexol clearance is a reference method for finding the glomerular filtration rate, which is important in nephrology practice and research. Several researchers had developed HPLC methods with UV detectors to determine plasma iohexol, but the chromatograms of iohexol using prior techniques showed two peaks of overlapping in iohexol isoforms. The improvement in the resolution of iohexol isoforms may give better data integration and minimize interference effects. Therefore, the aims of this study were to evaluate and compare a new reverse phase column, Alltech Alltima C18, with a previously described column for plasma iohexol determination. The plasma samples spiked with iohexol ex-vivo were extracted and separated by HPLC using 3 different columns. The performance of the standard separation method using Nucleosil C18 column was compared to Alltech Alltima C18 and Zorbax ODS columns. Retention times of exo-isoform peak from Alltech Alltima C18, Nucleosil C18 and Zorbax ODS were 9.41, 9.32, and 5.70 seconds, respectively. Alltech Alltima C18 produced the best characteristic chromatogram and symmetry peak and gave a linear calibration curve of standard iohexol concentration between 20-640 µg/ml (r^2 = 0.9997). Resolution of endo- and exo-isoforms, when analyzed by Alltech Alltima C18, Nucleosil C18, and Zorbax ODS were 1.5, 0.9, and 0.7, respectively. The percent recovery at 20, 300, and 600 µg/ml iohexol levels of Alltech Alltima C18, Nucleosil C18, and Zorbax ODS ranged from 99.9 - 100.3%, 93.3 - 100.2%, and 97.1 - 100.7%, respectively. Intra- and inter-assay precision (%RSD) of Alltech Alltima C18 were <2%. Only Alltech Alltima C18, the renal disease samples with creatinine >7.0 mg/dl, and the liver disease samples with bilirubin >27.3 mg/dl did not influence exo-isomer peak and percent recoveries of this method. Since Alltech Alltima C18 is a polymerically bonded and double end-capped column, it can improve the resolution of the iohexol isoforms by reducing the capacity factor of endo-isoform and increasing the capacity factor of exo-isoform. In conclusion, Alltech Alltima C18 is more suitable for iohexol determination than the other conventional RP columns, especially in the patient samples with renal and liver disease. This column produced simple, reliable, precise and accurate method and resulted in a clear resolution chromatogram.

KEY WORDS: GLOMERULAR FILTRATION RATE / IOHEXOL / PLASMA / CLEARANCE / HPLC-UV / END-CAPPED COLUMN

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การตรวจวิเคราะห์อิโอเฮกซอลในพลาสมาด้วยเทคนิคโครมาโตกราฟีของเหลวสมรรถนะสูง: ผลของการปรับปรุงวิธีใหม่ต่อการตรวจวิเคราะห์ในผู้ป่วยโรคไตและโรคตับ

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บทคัดย่อ

อัตราการกำจัดอิโอเฮกซอลออกจากร่างกายเป็นวิธีอ้างอิงสำหรับการหาค่าอัตราการกรองของไต ซึ่งมีความสำคัญต่อการบริหารจัดการผู้ป่วยโรคไตและการวิจัย นักวิจัยหลายท่านได้พัฒนาเทคนิคโครมาโตกราฟีของเหลวสมรรถภาพสูงที่ตรวจวัดด้วยเครื่องตรวจวัดอุลตร้าไวโอเล็ตสำหรับการตรวจหาปริมาณอิโอเฮกซอล แต่ลักษณะโครมาโตแกรมที่ได้ยังไม่สามารถแยกไอโซฟอร์มของอิโอเฮกซอลได้อย่างชัดเจน หากสามารถแยกไอโซฟอร์มได้ชัดเจนขึ้นอาจทำให้สามารถวิเคราะห์ข้อมูลได้ดีขึ้นและลดปัญหาการรบกวนจากสารอื่นได้ด้วย ดังนั้นจุดมุ่งหมายของการศึกษานี้คือ เพื่อประเมินและเปรียบเทียบการแยกอิโอเฮกซอลของคอลัมน์ Alltech Alltima C18 กับคอลัมน์มาตรฐานอื่น โดยอิโอเอกซอลจะถูกเติมลงในพลาสมา จากนั้นตัวอย่างจะผ่านกระบวนการเตรียมตัวอย่างและวิเคราะห์หาปริมาณอิโอเฮกซอลด้วยคอลัมน์ที่ต่างกัน 3 คอลัมน์ คุณลักษณะของคอลัมน์มาตรฐาน Nucleosil C18 จะถูกเปรียบเทียบกับ Alltech Alltima C18 และ Zorbax ODS จากผลการศึกษาพบว่าคอลัมน์ Alltech Alltima C18, Nucleosil C18 และ Zorbax ODS แยกเอ็กโซ-ไอโซฟอร์มของอิโอเฮกซอลออกมาที่เวลา 9.41, 9.32 และ 5.70 นาที ตามลำดับ โดยคอลัมน์ Alltech Alltima C18 ให้ลักษณะโครมาโตแกรมที่ดีที่สุดและมีความสมมาตรรวมทั้งให้กราฟมาตรฐานเป็นเส้นตรงในช่วงความเข้มข้น 20 ถึง 640 ไมโครกรัมต่อมิลลิลิตร ค่าการแยกกันระหว่างไอโซฟอร์มเมื่อวิเคราะห์ด้วย Alltech Alltima C18, Nucleosil C18 และ Zorbax ODS เป็น 1.5, 0.9 และ 0.7 ตามลำดับ และร้อยละของการคืนกลับของวิธีวิเคราะห์ที่ความเข้มข้นของอิโอเฮกซอล 20, 300 และ 600 ไมโครกรัมต่อมิลลิลิตรของ Alltech Alltima C18, Nucleosil C18 และ Zorbax ODS เป็น 99.9-100.3%, 93.3-100.2% และ 97.1-100.7% ตามลำดับ ความแม่นยำของวิธีการวิเคราะห์ภายในวันเดียวกันและต่างวันกันของ Alltech Alltima C18 มีค่าน้อยกว่า 2% มีเพียงคอลัมน์ Alltech Alltima C18 เท่านั้นที่พีคเอ็กโซ-ไอโซฟอร์มไม่ถูกรบกวนจากตัวอย่างของผู้ป่วยโรคไตที่มีระดับครีอตินิน มากกว่า 7.0 มิลลิกรัมต่อเดซิลิตร และผู้ป่วยโรคตับที่มีระดับบิลลิรูบินช่วงค่า 0.5-27.3 มิลลิกรัมต่อเดซิลิตร จากการที่ Alltech Alltima C18 เป็นคอลัมน์แบบโพลีเมอร์บอนด์และมีการทำดับเบิ้ลเอนแคปบนเม็ดซิลิกา ทำให้สามารถแยกไอโซฟอร์มของอิโอเฮกซอลได้ดีขึ้นโดยการทำให้เอนโด-ไอโซฟอร์มมีค่าความสามารถในการจับกับคอลัมน์ลดลงและเพิ่มค่าความสามารถในการจับกับคอลัมน์ของเอ็กโซ-ไอโซฟอร์ม ดังนั้น Alltech Alltima C18 จึงเป็นคอลัมน์ที่มีคุณสมบัติเหมาะสมมากที่สุดสำหรับการวิเคราะห์อิโอเฮกซอล นอกจากนี้การตรวจวิเคราะห์ยังง่าย มีความน่าเชื่อถือ ถูกต้องและแม่นยำ ให้สัมประสิทธิ์การแยกกันระหว่างไอโซฟอร์มได้ดีที่สุดด้วย

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LIST OF ABBREVIATIONS

HPLC	:	High Performance Liquid Chromatography
RP	:	Reverse Phase
CKD	:	Chronic Kidney Disease
ESRD	:	End Stage Renal Disease
CVD	:	Cardiovascular Disease
KDIGO	:	The Kidney Disease Improving Global Outcome Organization
GFR	:	Glomerular Filtration Rate
HPLC-MS/MS	:	High Performance Liquid Chromatography – Tendem Mass Spectrometry
CE	:	Capillary Electrophoresis
ICP-AES	:	Inductively Coupled Plasma – Atomic Emission Spectroscopy
CI	:	Confident Interval
SUN	:	Serum Urea Nitrogen
Cr	:	Creatinine
USG	:	Urine Specific Gravity
S	:	Substance
US	:	Urine concentration of Substance
PS	:	Plasma concentration of Substance
V	:	Volume of urine
^{51}Cr-EDTA	:	^{51}Cr-ethylenediaminetetraacetic acid
^{99m}Tc-DTPA	:	^{99m}Tc-diethylenepentaacetic acid
I	:	Iodine
ºC	:	Temperature in degree of Celsius
i.v	:	Intravascular
AUC	:	Area under the curve
IS	:	Internal Standard

LIST OF ABBREVIATIONS (cont.)

LC	:	Liquid Chromatography
UV	:	Ultraviolet
RI	:	Reflection index
IR	:	Infrared
NMR	:	Nuclear Magnetic Resonance
LS	:	Light Scattering
AR	:	Analytical Reagent
LOD	:	Lower Limit of Detection
LOQ	:	Lower Limit of Quantification
ICH	:	The International Conference on Harmonization on validation
RSD	:	Relative Standard Deviation
SD	:	Standard Deviation
CLSI	:	Clinical Laboratory Standards Institute
Rs	:	Resolution
t_R	:	Retention time
As	:	Asymmetry factor
k	:	Retention factor
$\bar{x}$	:	Mean
α	:	Selectivity
XRF	:	X-Ray Fluorescence
mg/dL	:	Milligram per deciliter
ml/min	:	Milliliter per minute
kg	:	Kilogram
ml/kg	:	Milliliter per kilogram
nm	:	Nanometer
v/v	:	Volume per volume
μm	:	Micrometer
mm	:	Millimeter

LIST OF ABBREVIATIONS (cont.)

ml	:	Milliliter
mg	:	Milligram
µg/ml	:	Microgram per milliliter

CHAPTER I
INTRODUCTION

1.1 Rationale / Problems

Chronic kidney disease (CKD) and end-stage renal disease (ESRD) are major health problems worldwide with dramatically rising incidence and prevalence (1, 2). Patients with CKD are at high risk of cardiovascular diseases (CVD) and cerebrovascular diseases (2). Therefore, the Kidney Disease Improving Global Outcome organization (KDIGO) has published clinical practice guidelines on the diagnosis, evaluation, prevention, and treatment of CKD. KDIGO recommended two laboratory tests for earlier diagnosis of CKD, glomerular filtration rate (GFR) and determination of proteinuria (3).

Determination of glomerular filtration rate plays an important role in nephrological practice, especially in diagnosis (4) and evaluation of risks of developing CKD (5). The gold standard for glomerular filtration rate (GFR) measurement is inulin. However, inulin clearance is difficult to perform, since it requires constant inulin infusion after single intravenous injection and inulin is not widely available. Plasma clearances of certain substances which are excreted by glomerular filtration have been used to determine GFR. The clearance of such substance produces compatible results to inulin clearance and is simple to perform. One such substance is iohexol. Since iohexol is not metabolized by the body, no toxic and freely filtered at the glomerulus, plasma iohexol clearance becomes a useful reference method for finding GFR.

Several methods have been developed to determine iohexol including high performance liquid chromatography with ultra violet detector (HPLC-UV) (6-8), high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) (9), capillary electrophoresis (CE) (10), inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (11). However, HPLC-UV assay is the most commonly

studied and superior to some other methods due to its available in laboratory and its flexibility.

When analyzed by HPLC-UV, iohexol exists the two isoforms, endo- (peak A) and exo-isoforms (peak B). Owing to exo-isoform has more ratio peak area than endo-isoform (about 80:20), peak B isoform is used for calculation due to produce the most accurate determination of iohexol by HPLC-UV (8, 9). Unfortunately, all the prior HPLC-UV studies showed the peak overlapping of iohexol isoforms (6-8). This might possibly cause of some inaccuracy data due to missing integration between the overlapping peaks. In addition, if the two isoforms could not be clearly separated, it might hard to assess the effect of unknown substances which may be the confounder in the chromatogram which leading to worst data integration.

Occasionally, iohexol clearance may be performed in patients with kidney failure or liver failure. As a matter of fact, uremic toxins accumulate in the blood of patients with renal failure and bilirubin or other toxins accumulate in liver failure. Such substances may interfere with HPLC results. The results from these samples may be altered by producing confusing peaks in the chromatogram. The interference may be less significant if the resolution of the iohexol isoforms is improved.

Column selection is a tool to obtain good separation. Since the different column properties result in the different resolutions, improvement of the resolution of iohexol isoform peaks may achieved by choosing the suitable column. The previous studies had performed methods by using Nucleosil C18 (7), Lichrospher (8) or µBondapak C_{18} analytical columns (6). All these conventional columns could not present clearly separation between peak A and B isoforms. Therefore, the aim of this study is first to introduce a new reverse phase column, Alltech Alltima C18 that has a different phase bonding and double end-capping on column packing materials, for plasma iohexol determination by comparing column performance and method validation parameters with previously described column. Secondly, the effects of renal disease and icteric plasma on a new improvement plasma iohexol determination are assessed.

CHAPTER II
OBJECTIVES

2.1 Objectives

To improve the reverse phase HPLC-UV method which can quantitate iohexol concentration in healthy subject and various diseases patient plasma for estimating GFR with a simple, accurate, precise, sensitive and reliable technique.

2.2 Specific objectives

1) To develop and optimize the analytical method for iohexol determination.
2) To validate the analytical method among three columns.
3) To compare column performance of three columns.
4) To assess an interference of high creatinine plasma from renal disease patients onto the analytical method.
5) To assess an interference of high bilirubin plasma (icteric plasma) from liver disease patients onto the analytical method.

CHAPTER III
LITERATURE REVIEW

3.1 Chronic renal diseases as a public health problem

Chronic kidney disease (CKD) is a major health concern worldwide (12). CKD is often a progressive disorder as the kidney function usually declines eventually leading to end-stage renal disease (ESRD). Such patients require treatment with dialysis or transplantation, which are expensive and associated with high morbidity and mortality. In addition to ESRD, patients with CKD have marked increase in risk of cardiovascular disease which is in direct relation to the severity of CKD (4, 12, 14).

In 2002, the US Kidney disease outcome Quality Initiative (K/DOQI) committee proposed that CKD should be diagnosed and classified into 5 different stages by the presence of proteinuria, and on glomerular filtration rate (GFR) as an indicator of kidney function (15). These recommendations have been accepted worldwide in the nephrological community, and were endorsed in 2004 by the independent international Kidney Disease Improving Global Outcome organization (KDIGO). In 2009, the staging of CKD has been further modified by the KDIGO, but GFR remain the cornerstone for diagnosis and classification of CKD (Table 3.1) (16, 17). In the recent analysis, both the risks of progressing to ESRD and the risks of developing cardiovascular disease or death are dependent on the stages of CKD, which in turn is dependent on the GFR.

Table 3.1 Stages of chronic kidney disease.

Stage	Description	GFR (ml/min per 1.73m^2)
1	Kidney damage with normal or ↑GFR	GFR ≥ 90
2	Kidney damage with mild ↓ GFR	60 – 89
3	Moderate ↓ GFR	30 – 59
4	Severe ↓ GFR	15 – 29
5	Kidney failure	<15 (or dialysis)

Around the world, the incidence and prevalence of CKD and ESRD has increased dramatically (3). Coresh J et al. (18) found the prevalence of CKD in US adults aged 20 years or older between 1999 - 2004 has increased by 30% compared to between 1988 - 1994 (Table 3.2).

Table 3.2 Prevalence of chronic kidney disease (CKD) stage in US adults aged 20 years or older based on NHANES 1988 - 1994 and NHANES 1999 - 2004.

CKD Stages	Prevalence, % (95% CI)		Prevalence ratio for NHANES 1999 - 2004 to 1988 – 1994
	NHANES 1988 - 1994	NHANES 1999 - 2004	
1	1.71 (1.28 - 2.18)	1.78 (1.35 - 2.25)	1.05 (0.85 -1.30)
2	2.70 (2.17 - 3.24)	3.24 (2.61 - 3.88)	1.21 (1.03 - 1.41)
3	5.42 (4.89 - 5.95)	7.69 (7.02 - 8.36)	1.42 (1.25 - 1.62)
4	0.21 (0.15 - 0.27)	0.35 (0.25 - 0.45)	1.70 (1.11 - 2.51)
5	*NA*	*NA*	*NA*
Total	**10.03 (9.16 - 10.91)**	**13.07 (12.04 - 14.10)**	**1.30 (1.19 - 1.43)**

Levey et al. (13) showed that the incidence and prevalence of ESRD patients who are treated with dialysis and transplantation in the US have nearly doubled from 340,000 in 1999 to 651,000 in 2010. Data from many series have shown that Thailand has among the highest rates of CKD in the world (19, 20, 21). The rates of CKD are between 4-12%. The numbers of patients with ESRD requiring renal replacement therapy (RRT) has also risen dramatically. The prevalence from by more than 15 folds from 30 to 507 per million population in 11 years from 1998 to 2010.

Diabetes and hypertension are important risk factors, but in many cases the causes of CKD are unexplained. Unlike many clinical conditions, patients with CKD are often asymptomatic in the earlier stages. In the late stages, patients may develop nausea, anorexia and coma due to build up of uremic toxins. Furthermore, patients with CKD are at high risk of cardiovascular diseases and cerebrovascular diseases. Early diagnosis of CKD is important as at this time intervention to slow kidney disease progression is most effective. Because of the lack of symptoms, diagnosis of CKD

relies on laboratory measurements. Accurate assessment of GFR is critical to identify and monitor patients with CKD such that they can be adequately treated to stop progression of CKD to end-stage renal disease and to lower the enormous cardiovascular co-morbidity associated with CKD (3).

3.2 Glomerular filtration rate (GFR)

The GFR is a measure of the efficiency of which substances are cleared from the blood by glomerular filtration, and is defined as the volume of plasma from which a given substance is completely cleared by glomerular filtration per unit time. GFR is the best clinical estimate of functioning renal mass, and correlate well with the clinical severity of renal function disturbance (22). GFR cannot be measured directly in humans. Rather, it has to be determined by measuring the clearance of an ideal filtration marker administered to the body exogenously or estimated from plasma concentrations of endogenous markers of retained waste product. A variety of exogenous (radioisotopic and non-radioisotopic) and endogenous markers have been used to measure or estimate GFR.

3.2.1 Endogenous GFR markers

In routine clinical practice, blood urea nitrogen (BUN) and serum creatinine (Cr) concentrations are the most commonly utilized endogenous markers of renal function as measurements of retention of renal nitrogenous wastes. Both serum creatinine and urea vary inversely with GFR, there is marked inter- and intra-individual variations in this relationship. Urea is major nitrogenous end product of protein and amino acid catabolism, produced by liver and distributed throughout intracellular and extracellular fluid. In the kidneys, urea is filtered out of blood by glomeruli and is then partially reabsorbed with water. Urea clearance is an inaccurate marker of glomerular filtration rate as its overproduction rate is not constant and depends on several non renal factors, including diet protein intake and urea cycle enzymes, hydration status. As such, BUN alone is not an accurate method to determine GFR and is only used as an adjunct to renal function estimate with serum creatinine.

Creatinine is a byproduct of hydrolysis of creatine phosphate in muscle. Creatinine is produced at relatively constant rates in the normal individuals although the rates of production may vary widely between individuals and during illness. To account for variations in muscle mass and hence creatinine generation, GFR estimating equations based on serum creatinine with coefficients for age, sex , body weight or race have been devised. Nonetheless, even with these formulae such as the MDRD or Cockcroft-Gault, the deviation of GFR estimated from serum creatinine from measured GFR can be as high as 30%.

Furthermore, serum creatinine concentrations are affected by muscle damage, with elevated concentrations occurring during rhabdomyolysis. In addition loss of muscle mass as in severe emaciation or advanced liver disease may cause low serum creatinine concentrations leading falsely overestimating kidney function. Creatinine is freely filtered by the glomerulus, but there is significant tubular secretion. The proportion of tubular secretion to filtered creatinine varies widely between individuals and may be higher in patients with CKD. As such serum creatinine concentrations do not elevate until greater than 75% of GFR has been affected and therefore, creatinine is a relatively insensitive method of assessing early or minor renal dysfunction.

Creatinine can be assayed in several ways but the most frequently utilized is the Jaffe method, a colorimetric assay based on the formation of a complex between creatinine and alkaline picrate. Non-creatinine chromagens (glucose, pyruvate, acetoacetate, fructose, uric acid, and ascorbic acid) in the serum which are also measured by the Jaffe assay may contribute to up to 20% of the measured serum creatinine concentration causing overestimation of the true creatinine concentration. As serum creatinine concentrations elevate, the proportion of non-creatinine chromogens decreases and the total value becomes more accurate.

Although endogenous markers of renal function such as serum creatinine or creatinine based GFR equations are convenient to use in clinical practice, they are not sensitive to early or small changes in renal function, and are plagued by inaccuracies. Accurate measurements of GFR would allow more appropriate monitoring of renal function especially in patients with malnutrition or liver disease, who have disturbed creatinine generation.

3.2.2 Exogenous markers

Quantitative measures of renal function can be categorized as plasma disappearance curves or clearance studies involving timed urine collections. In order to reflect GFR, the ideal glomerular filtration marker substance must meet the following requirements: 1) freely filtered by the glomerulus, 2) no significant renal tubular secretion or reabsorption, 3) no significant binding to plasma protein, 4) non-toxic and 5) not significantly metabolized by the body. Of these, the fructose polymer inulin best stratifies the above criteria and renal (urinary) inulin clearance has been regarded as the most accurate (gold standard) method to measure GFR (23). Inulin is not synthesized nor metabolized by the body, and is not reabsorbed or secreted by the kidney. Therefore, the amount of that inulin (I) filtered at the glomerulus is equal to the amount excreted in the urine. The amount of I filtered at the glomerulus equals GFR multiplied by plasma I concentration: GFR X P_I. The amount of I excreted equals the urine S concentration (U_I) multiplies by the urine flow rate (V, volume excreted per unit time).

Since filtered I = excreted I

GFR X PS = US X V

GFR = $(U_I \times V)/P_I$

Where GFR = clearance in unit of ml, of plasma cleared of the substance per minute, P_I = plasma concentration of the substance.

While inulin clearance is still considered the gold standard for GFR measurements, it is difficult to perform in practice. Inulin is not widely available and difficult to get into solution. To dissolve inulin, it is necessary to heat inulin to 95C. Urinary inulin clearance also requires constant infusion of inulin to ensure that a stable plasma level has been achieved. Urine collections are also difficult to perform and also require patients to be able to empty their bladders completely.

As a consequence of the difficulty to perform urinary inulin clearance, the plasma clearance of radioactive, and non-radioactive exogenous markers have been used instead to measure GFR. Plasma clearance of radioactive agents including ^{125}I-iothalamate, ^{51}Cr-ethylenediaminetetra acetic acid (^{51}Cr-EDTA), ^{99m}Tc-diethylenetriaminepenta acetic acid (^{99m}Tc-DTPA) are considered similar to inulin clearance and have recently been endorsed by the British Nuclear Medicine Society as

standard measures of GFR (22). Unfortunately, problems associated with handling radioactive materials and the risks of exposure to humans pose limits on their use. Recently, plasma clearance of non-radioactive iohexol after single bolus injection has gained increasing acceptance for the determination of GFR (24, 25). Iohexol fulfill characteristic necessary criteria to determine GFR and iohexol clearance is comparable to inulin clearance (9) and iohexol clearance technique have even been applied to capillary blood/filter paper collection system, which is attractive alternative for community based field study (7).

3.3 Role for accurate and sensitive GFR measurements

Accurate methods to measure GFR such as iohexol clearance have many potential applications in both routine clinical practice and in research. By identifying patients with minor degrees of reductions in GFR early (before serum creatinine is abnormal), it offers the ability of early intervention for CKD, and prevention of subsequent decline in renal function. Accurate analysis of GFR allows for correct staging of kidney disease and enables the success of therapy either in the routine clinic or in therapeutic trials to be measured reliably. GFR assessment is an integral part of kidney transplant donor analysis and evaluation of patients with cirrhotic liver disease. Iohexol clearance has been used in many studies to evaluate the prevalence of CKD, to ascertain CKD staging, and to study kidney function in different populations.

3.4 Iohexol

3.4.1 Introduction

Iohexol is a non-ionic, water-soluble radiographic contrast medium with a molecular weight of 821.14. In aqueous solution each triiodinated molecule remains undissociated. Iohexol is commonly used in adults and children as radiocontrast agent for urography contrast enhanced computed tomography and angiography. Once injected, iohexol is not metabolized by the body, bound to plasma proteins, secreted or

absorbed by the renal tubules, and is freely filtered at the glomerulus, making it a useful marker for GFR studies (26).

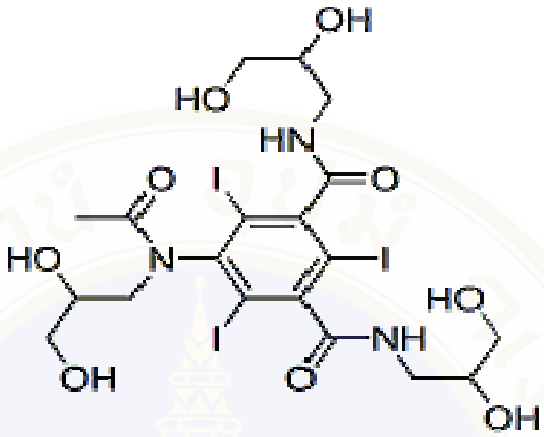

Figure 3.1 Iohexol structure.

3.4.2 Physical and chemical properties

Chemical name:	N,N´ - Bis (2,3-dihydroxypropyl)-5-[N-(2,3-dihydroxypropyl) acetamido]-2,4,6 triiodoisophthalamide
Generic name	: Iohexol
Trade name	: Omnipaque
Molecular formulae	: $C_{19}H_{26}I_3N_3O_9$
Molecular weight	: 821.138 g/mol
Melting point	: 174-180 ºC
Boiling point	: 892 ºC
pKa	: 11.35

3.4.3 Pharmacokinetics

Immediately following rapid intravascular injection, Omnipaque (iohexol) reaches peak plasma concentration and is then rapidly distributed throughout the extracellular fluid compartment. Iohexol does not normally cross the blood-brain barrier to any significant extent. It is excreted unchanged by the kidneys, mainly by glomerular filtration; tubular secretion plays a minor role, and a very small quantity

(1-2%) is excreted via the bile. About 80-90% of the injected dose is excreted in the first 24 hours, with peak urine concentrations occurring in the first hour.

Pharmacokinetic studies of iohexol following i.v. injection in healthy male volunteers showed, using a three-compartment open model, that its distribution half-life is 22 minutes, excretion half-life 2.1 hours, and first-order terminal elimination half-life 12.6 hours. The volume of distribution of the central compartment is 165-270 ml/kg, the mean renal clearance 120 ml/min., and the mean total body clearance is 131 ml/min.

In the presence of impaired renal function, the excretion of iohexol by the kidneys will be delayed and the amount excreted in the bile increases. Iohexol is not known to be appreciably metabolized in humans. No metabolites have been found in urine. The presence or absence of metabolites in human bile has not been ascertained (small quantities of two metabolites were detected in rabbit bile and urine).

3.4.4 Toxicity and adverse reaction

Undesirable effects associated with the use of iodinated contrast media are usually mild to moderate and transient in nature, and less frequent with non-ionic than with ionic contrast media. Serious reactions as well as fatalities are only seen on very rare occasions. The most frequent adverse event is a mild, general sensation such as a feeling of warmth or a transient metallic taste. Abdominal discomfort/pain and gastrointestinal reactions like nausea, vomiting and diarrhea may occur. Hypersensitivity reactions are rare and usually present as mild respiratory or coetaneous symptoms like dyspnoe, rash, erythema, urticaria, pruritus and angioedema. They may appear either immediately after the injection or up to a few days later. Severe manifestations such as laryngeal edema, bronchospasm or pulmonary edema are very rare. Severe and even toxic skin reactions have been reported. Headache or fever may occur. Episodes of hypertension may also occur. Pyrexia with rigors is seen on rare occasions.

3.5 Analytical method for determination of iohexol

Varieties of methods have been to apply to determination of iohexol such as a colorimetric including deiodination of iohexol by alkaline hydrolysis and a measurement a release of iodine by the ceric arcernite method (27), X-ray fluorescent (28), capillary electrophoresis (10) and inductively coupled plasma atomic emission spectroscopy (11).

3.5.1 High performance liquid chromatography-ultraviolet detector (HPLC-UV)

Several HPLC-UV methods have been introduced to determine iohexol many years ago and now it is the most popular method for determine iohexol. The commercially available preparation of iohexol contains two isomers of the substance (6, 8). When examined in serum or urine by HPLC, the two isomers of iohexol have been shown to have difference retention times. This complicates pharmacokinetic calculations in which the iohexol plasma clearance is calculated by dividing the dose of substance by area under the curve (AUC) of the plasma levels of iohexol. In order to perform such a calculation, the amount of iohexol isomers in the pharmaceutical preparation has to be known. An alternative would to be measure the plasma level of only one iohexol isomer and to calculate the clearance form the volume of distribution and the elimination haft-life. However, this requires the use of a pharmacokinetic model. Usually a two-compartment model is use to describe the time-cause of the plasma level of iohexol after intravenous injection of the compound.

In 1995, Gaspari and colleague (24) determined iohexol in plasma by using HPLC-UV, elution of iohexol from chromatographic column was carried out using 4% acetonitrile in water and adjusted pH to 2.5 as mobile phase and analytical column use to elute is Lichrosorb C-18 column.

In 2005, Soman and colleague (6) developed and validated HPLC-UV method to determine iohexol in plasma, elution of iohexol and its IS from the chromatographic column was carried out using a fast gradient elution technique. The composition of mobile phase was rapidly changed (within 6s) from an initial 4% acetonitrile to 14% acetonitrile in water (v/v), after which the composition was

changed back to 4% acetonitrile comprising a total run time of 12 min and analyzed by Bondapak C_{18} analytical column (150.0 mm X 3.9 mm, 10 μm particle size).

In 2006, Nicelusco-Davaz and colleague (7) developed technique to determine iohexol from finger-pick by used 3.5% acetonitrile in water (v/v) as mobile phase, pH at 2.5 to elute iohexol and analyzed by Nucleosil C18 column (250 mm X 4.6 mm, 5 μm particle size).

Schwartz GJ and colleague (25) determined iohexol in plasma by HPLC-UV, iohexol was eluted by isocratic elution with 20 Mn potassium phosphate pH 2.5 and 4% acetonitrile; the analytical column was Zorbax Eclipse XBD-C8 4.6 X 150 mm, 5 mm particle size column and maintained at 30 ºC.

These HPLC-UV methods shown different performance such as different in retention time, different in resolution, different retention factor and that can be a cause of difference in precision, accuracy of these methods.

3.5.2 High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS)

High-performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) has been used to determine iohexol. Although this method provides many advantages over than other methods, but it is cost-effective and requires special technician. Lee et al (9) used HPLC-MS-MS to determine iohexol in serum and they obtained recoveries in acceptable range and good precision both inter- and intra-days.

3.5.3 Capillary electrophoresis

Capillary electrophoresis is alternative technique to determine iohexol in plasma. Michael VR et al (10) used this technique to determined iohexol in plasma and they found that this technique was accurate as HPLC technique. CE has many advantages such as CE not requires the use of expensive column, mobile phase gradient, and solvent pump. Consumable supplies for CE is less costly than HPLC and this method requires short time to determine iohexol.

3.5.4 Inductively coupled plasma atomic emission spectrophotometer

Braselton et al (11) introduced inductively coupled plasma atomic emission spectrophotometer to measure iodine in molecule of iohexol. Iodine was determined at 178.276 nm on the phosphorus 178.287 nm channel of the polychromator by utilization of spectrum shifter offset software, while correcting for P with the sequential P 214.914 nm emission line.

3.6 Endo-exo isomerism

Endo-exo isomerism is a special type of isomerism found in organic compounds with a substituent on a bridge ring system. The prefix endo is reserved for the isomer with the substituent located closest, or "syn" to the longest bridge. The prefix exo is reserved for the isomer with the substituent located furthest, of "anti", to the longest bridge. Here "longest" and "shortest" refer to the number of atoms that comprise the bridge. The type of molecular geometry is found in norbornene systems such as dicyclopentadiene.

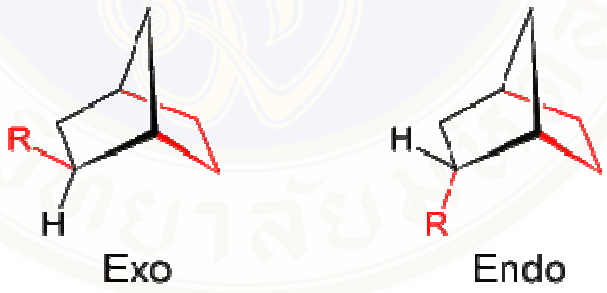

Figure 3.2 Structures of exo- and endo- iohexol isomers.

For endo- and exo-isomers of iohexol, endo-isoform occurs in minor amount while exo-isoform occurs in major amount (about 20: 80) (6).

3.7 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a separation technique utilizing differences in distribution of compounds to two phases, called mobile phase and stationary phase. The mobile phase is when the sample dissolved in a solvent which is the force though a column at high pressure, the stationary phase is when the

particle held inside the column interact with the mobile phase and retard the passage of the different components of the sample to differing degree.

HPLC becomes a very popular in the field of analytical chemistry for the following reasons: it can be run at ambient temperature; it is non destructive to the compound of interest, which can be collected intact; in many instances, derivatization is not necessary for response; and column can be loaded with large quantities of the material for determination of low level.

The HPLC principle base on separation technique, mobile phase carrying a mixture is caused to move in contact with a selectively absorbent stationary phase. Different components in the sample are carried forward at different rate by moving liquid phase according to their differing interactions with the stationary and mobile phases. In a liquid chromatographic process, a liquid permeates through a porous solid stationary phase and elutes to solute into a flow-through detector.

HPLC system consists of mobile phase, pump, injector, separation column, detector and data acquisition as shown in Figure 2.2.

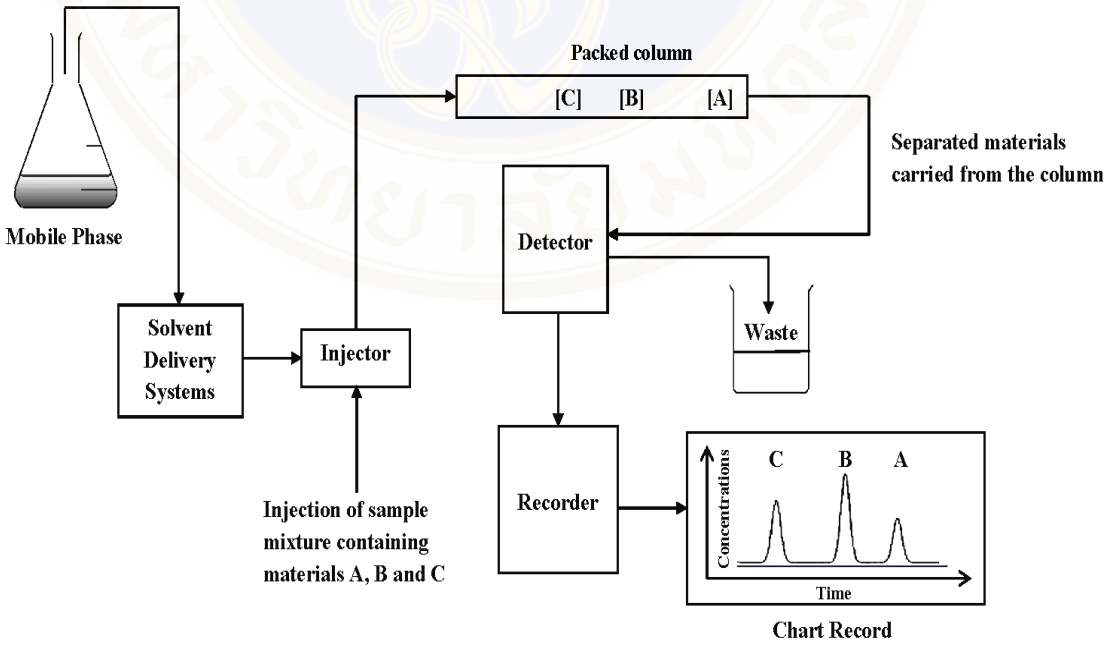

Figure 3.3 Scheme of a basic high performance liquid chromatography system.

3.7.1 Injector

The injector serves to introduce the liquid sample into the flow stream of the mobile phase. It lies between the pump and the column; it also must be able to operate at high pressures. Injectors work on the principle of the loop and valve. In the load position, system solvent is displaced from the injection loop with sample solution at atmospheric pressure. Sample goes into the loop at the point closest to the column and displaces loop solvent out the back end of the loop. Injection occurs by roating the injector against a Teflon® seal to the inject position. This places the loop containing the sample in the solvent flow from the pump to the column. Since mobile phase enters the loop at the end opposite sample injection, first-out loading of the sample onto the column head. No dilution of the sample solution occurs within the sample loop solvent volume. The loop is pressurized and the sample is washed onto the column. Loop volume can range between 10 µl to over 500 µl. In modern HPLC system, the sample injection is typically automated.

3.7.2 Mobile phase

Mobile phase in HPLC refers to the solvent being continuously applied to the column and acts as a carrier for sample solution. Sample solution is injected into the mobile phase of an assay through injector port. As a sample solution flows through the column with a mobile phase, the components of that solution migrate according to the non-covalent interactions of the compound with the column. The chemical interaction of mobile phase and sample with the column, determination the degree of migration and separation of components contained in the sample. For example, those samples which have stronger interactions with the mobile phase than with stationary phase will be eluted from the column faster and thus have shorter retention time. The mobile phase can be altered in order to manipulate the interactions of the sample and the stationary phase.

3.7.3 Pump

Pumps are basically devices for pulling in solvent, pressurizing it, and driving it out through the injector, column, and detector at high pressure. There are

several types of pump available for use with HPLC analysis, they are; syringe type pumps, reciprocating pistal pumps and constant pressure pumps.

3.7.4 Guard columns

A guard column is a small column placed before the analytical column to protect it from particles or contaminants from the samples. Ideally, guard columns are packed with the same materials as analytical columns and should not cause a significant increase in pressure or performance degradation. Guard columns are commonly used in applications involving dirty samples environmental or bioanalytical samples where more extensive sample clean-up might not be feasible. Some users might prefer the use of an in-line filter, though it does not have the sample capacity as a well- designed guard column. Another type of guard column, termed a scavenger column, is placed between the pump and the injector. Its purpose is to protect the analytical column from mobile phase contaminants. Its use can be desirable for small particle columns, which are easily plugged, and in ion-pair chromatography to pre-filter the mobile phase.

3.7.5 Column

The HPLC column is contained with stationary phase, which refers to solid phase to support the mobile phase continuously flows that provides differential of sample components. A typical HPLC column is a stainless steel tube filled with small-particle packing used for sample separation. HPLC columns can be categorized in a number of ways: by column hardware, by dimensions and by support types. The column containing various types of stationary phases are commercially available. Reversed phase (RP) columns are most used for many liquid chromatographic (LC) methods.

3.7.6 Detector

An HPLC detector measures the concentration (or mass) of eluting analytes by monitoring one of their inherent properties, such as UV absorbance. A detector can be universal to all analytes or specific to particular classes of analytes.

Early HPLC detectors are spectrometers equipped with small flow cells, however most modern units are compact and designed solely for HPLC.

There are many types of detectors that can be used with HPLC such as refractive index (RI), ultraviolet (UV), Fluorescent, radiochemical, electrochemical, near infrared (near IR), mass spectrometer, nuclear magnetic resonance (NMR) and light scattering (LS). Ultraviolet (UV) detector is absorbance detector monitors the absorption of UV or visible light in the HPLC eluent. It is the most common detector since most analytes of interest have UV absorbance properties. A UV detector consists of a deuterium lamp, a monochromator, and a small flow cell. A monochromator consists of a movable grating or prism that allows the selection of a specific wavelength to pass through the exit slit. A dual-beam optical design is common. Here the light source is split into a sample and a reference beam, and the intensity of each beam is monitored by a separate photodiode. Only the sample beam passes through the sample flow cell. A flow cell has typical volumes of 2-10 µl and path lengths of 210 mm, with quartz lenses serving as cell windows.

3.7.7 Data acquisitions

Analytical results are calculated from measurements made directly on the chromatogram provided by the chart record by converting the detector signal to a picture chromatogram and provides manual by automated determination of the identifiable and amounts of sample components.

3.8 Role of stationary phase to separation

The column is the heart of the HPLC system. It holds the support media for the stationary phase that provides differential retention of sample components. The many different separation mechanisms in HPLC column, based on e.g. hydrophobic, hydrophilic and ion-pairing interactions, and size exclusion effects together with the availability of a large number of high quality stationary phases, explain its great popularity (29). The large number of columns was introduced to the market; unfortunately the trade name of these supports does not sufficiently describe their chromatographic behavior. Factors such as particle size, surface area, pore size, trace

metal activity, bonded phase surface activity, bonding chemistry, silica deactivation process can all influence retention selectivity and peak shape properties of analytes. All these variables will result in insignificant differences in chromatographic performances among packings as well as batch differences for a given packing. Column selection is important in method development but selection of columns with suitable selectivity for a given analysis is difficult. Ideally, column selection should be based on objective criteria. In other words, columns should be characterized in order to facilitate proper column selection for each specific application. In routine practice, changing mobile phase to obtain good separation is commonly but it tedious and time consuming. The easy way, changing column to change selectivity is quickly and surprisingly powerful. Selecting the right column can often decrease the total run time and can change peak elution order for the analysis of samples containing both non-polar and polar compounds and the right column give more precise and accurate than others.

3.8.1 Packing material

3.8.1.1 Base Materials

Silica-based packing is compatible with a wide range of aqueous and organic solvents. Silica-based column can withstand high pressure. Most silica is stable from pH 2-7.5 but special silica may stable from pH 1-10. Silica provides high resolution or sharp peaks with small molecules. Silica-based columns are often used for separation of low molecular weight analytes. Polymer-based packing is compatible with most mobile phase solvents and samples with a pH 1-14. It is lower efficiencies for a small molecules compared to silica-based due to smaller surface area. Polymer-based columns are often used for ion exchange or ion exclusion chromatography.

3.8.1.2 Particle Size

Standard particle size is 5 μm. The smaller particle sizes give higher efficiency and higher resolution than larger particle sizes. However, larger particle sizes offer faster flow rates and lower back-pressure.

3.8.1.3 Pore Size

In general, packing materials with a smaller pore size provide higher surface areas and higher capacities than packing materials with larger pore sizes. For general purposes of reverse-phase application, pore size 100-120 μm is recommended. For higher resolution and large molecule such as proteins, pore size 60-80 μm and 300 μm are recommended, respectively.

3.8.1.4 Surface area

A larger surface area offers higher capacity and greater resolution however smaller surface area equilibrates faster.

3.8.1.5 Phase Type

There are 2 types of bonding, polymeric and monomeric. Polymeric bonding offers better column stability under aggressive mobile phase. Monomeric bonding offers lower back pressure. However, high-purity silica phases are very stable whether monomeric or polymeric bonding.

3.8.1.6 End-capping

Free silanol in silica-based reversed-phase packings will interact with polar compounds. End-capping the bonded phase with C2-C4 will minimize these interactions. However, non-end-capped phases enhance polar selectivity and stronger retention of polar organic compounds.

3.8.1.7 Carbon load

Lower carbon loads are more weakly hydrophobic and reduce retention times. Higher carbon load offers higher capacity and greater resolution.

3.8.2 Column information

3.8.2.1 Alltech Alltima reversed-phase column

Alltech Alltima reversed-phase column is a line of HPLC columns in the Alltech HP family. The Alltech Alltima family is high quality general purpose columns. It is made with spherical silica, polymerically bonded and double end-capped for long lifetimes, even when using harsh mobile phase. It generates symmetrical peaks even for difficult acid- and amine-containing compound. Proprietary acid and base deactivation processes eliminate activated silinols, the primary source of tailing on silica column.

3.8.2.2 Nucleosil C18 reverse-phase column

Nucleosil C18 reverse-phase column is packing used for reverse-phase liquid chromatography. It is made with spherical silica, monomerically bonded and single end-capped. This packing is produced by chemically bonding C18 group on silica particle. It has a 25% carbon load for strong retention.

3.8.2.3 Zorbax ODS reverse-phase column

Zorbax ODS is packing used for reverse phase liquid chromatography. This packing is produced by chemically bonding octadecylsilane group to Zorbax SIL silica particles. The ODS bonded phase is monolayer coating produced by reacting a monofunctional silane with the Zorbax support. The coated 5-6 μm Zorbax particles are spherical in shape and uniform size to give optimum column efficiency. Columns are loaded to a uniform bed density using a proprietary high-pressure slurry loading technique. Since optimum column performance is directly related to uniform bed density, Zorbax ODS is offered in pretested columns.

3.8.3 Column efficiency parameters

3.8.3.1 Retention factor (*k′*)

The retention factor (*k′*) is the degree of retention of the sample component in the column. *k′* is defined as the time the solute resides in the stationary phase (t) relative to the time it resides in the mobile phase (t). *k′*, an IUPAC term, was often referred to as k or capacity factor in many references.

Retention factor, $k = (t_R - t_M)/t_M$

Where; t_R = retention time of isomer B of iohexol

t_M = void time (retention time of uracil)

A peak with k = 0 is a component that is unretained by the stationary phase and elutes with the solvent front. k >20 indicates that the component is highly retained. In most analyses, analytes elute with k between 1 and 20 so that they have sufficient opportunity to interact with the stationary phase resulting in differential migration. Analytes eluting with k > 20 are difficult to detect due to excessive band broadening.

3.8.3.2 Separation factor (α)

The separation factor or selectivity (α) is a measure of relative retention k'_1/k'_2 of two sample components. Selectivity must be >1.0 for peak separation. Selectivity is dependent on many factors that affect k' such as the nature of the stationary phase, the mobile phase composition, and properties of the solutes.

Selectivity (α) = k'_B / k'_A

Where; k'_B = capacity factor of second peak

k'_A = capacity factor of first peak

3.8.3.3 Column Efficiency and Plate Number (N)

An efficient column produces sharp peaks and can separate many sample components in a relatively short time. As seen in most chromatograms, peaks tend to be Gaussian in shape and broaden with time, where w_b becomes wider with longer t_R. This band broadening inside the column is fundamental to all chromatographic processes. The number of theoretical plates or plate number (N) is a measure of the efficiency of the column. N is defined as the square of the ratio of the retention time divided by the standard deviation of the peak (σ)

$$N = \left(\frac{t_R}{\sigma}\right)^2 = \left(\frac{2.355 t_R}{W_{1/2}}\right)^2 = 5.546\left(\frac{t_R}{W_{1/2}}\right)^2$$

Where : t_R = retention time

$W_{1/2}$ = half of peak width

σ = standard deviation

3.8.3.4 Resolution (R_s)

The goal of most HPLC analyses is the separation of one or more analytes in the sample from all other components present. Resolution (R_s) is a measure of the degree of separation of two adjacent analytes. R_s is defined as the difference in retention times of the two peaks divided by the average peak width.

Ideally, the goal of most HPLC methods is to achieve baseline separation (R_s = 1.2 - 2.0) for all key analytes.

$$R_s = \frac{2[(t_R)_B - (t_R)_A]}{W_A + W_B}$$

Where; t_{RA} = retention time of first peak

t_{RB} = retention time of second peak

W_A = peak width of first peak

W_B = peak width of first peak

3.8.3.5 Asymmetry factor (A_s)

The asymmetry factor (A_s) is used to measure the degree of peak symmetry and is defined at peak width of 10% of peak height (W).

$$A_s = \frac{(A + B)}{2B}$$

3.9 Method validation

A number of guidance documents on method validation have been issued by various international organizations and conferences (30). Analytical test method validation is completed to ensure that an analytical methodology is accurate, specific, reproducible and robust over the specified range that an analyte will be analyzed. Method validation provides an assurance of reliability during normal use. Unfortunately, there is no single source or final guideline on analytical method validation. Validation is customized by choosing necessary tests and acceptance criteria for a given method. The comprehensiveness of this kind of validation is based upon the type of method and its requirements.

3.9.1 Validation parameters

According to the international conference on harmonization on validation (28) of analyte procedure in 1994, there's a general agreement, that at least the following validation parameters should be evaluated: selectivity, calibration model (linearity), stability, accuracy (bias), precision (repeatability, intermediate precision) and the lower limit of quantification (LOQ). Additional parameters which may be relevant include limit of detection (LOD), recovery, reproducibility, and ruggedness (robustness) (27). For qualitative procedures, a general validation guideline is currently not available, but there seems to be agreement that at least selectivity and the LOD should be evaluated and that additional parameters like precision, recovery and ruggedness might also be important.

3.9.1.1 Selectivity

Selectivity is defined as the ability of the analytical method to measure unequivocally and to differentiate the analyte (s) in the presence of components, which may be expected to be present. Typically, these might include metabolites, impurities, degradants, matrix components, etc. It should be noted that the term specificity is often used interchangeably with selectivity, although in a strict sense this is not correct.

3.9.1.2 Calibration model (linearity)

The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.

3.9.1.3 Working range

The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.

3.9.1.4 Accuracy

Accuracy is the closeness of the test results obtained by the analytical method to the true value. Accuracy is usually determined in one of four ways. First, accuracy can be assessed by analyzing a sample of known concentration (reference materials) and comparing the measured value to the true value. The second

approach is to compare test results from the new method with results from an existing alternate well characterized procedure that is known to be accurate. The third approach, based on the recovery of known amounts of analyte, is performed by spiking analyte in blank matrices. For assay methods, spiked samples are prepared in triplicate at three levels over arrange of 50–150% of the target concentration. The fourth approach is the technique of standard additions, which can also be used to determine recovery of spiked analyte. This approach is used if it is not possible to prepare a blank sample matrix without the presence of the analyte. This can occur, for example, with lyophilized material, in which the speciation in the lyophilized material is significantly different when the analyte is absent.

3.9.1.5 Precision

Precision is the measure of the degree of repeatability of an analytical method under normal operation and is normally expressed as the percent relative standard deviation (RSD) for a statistically significant number of samples. According to the ICH, precision should be performed at three different levels: repeatability, intermediate precision, and reproducibility. Repeatability is the results of the method operating over a short time interval under the same conditions (intra-assay precision). It should be determined from a minimum of nine determinations covering the specified range of the procedure (for example, three levels, three repetitions each). Intermediate precision is the results from within-laboratory variations due to random events such as different day, analysts, equipment, etc. In determining intermediate precision, experimental design should be employed so that the effects (if any) of the individual variables can be monitored. Precision criteria for an assay method is that the instrument precision (RSD) will be ≤1% and the intra-assay precision will be ≤2%. Reproducibility, which is determined by testing homogeneous samples in multiple laboratories, is often a part of inter-laboratory crossover studies. The evaluation of reproducibility results often focuses more on measuring bias in results than on determining differences in precision alone. Statistical equivalence is often used as a measure of acceptable inter-laboratory results. An alternative, more practical approach is the use of “analytical equivalence”, in which a range of acceptable results is chosen prior to the study and used to judge the acceptability of the results obtained from the different laboratories.

Documentation in support of precision studies should include the SD, RSD, coefficient of variation, and the confidence interval. Reproducibility is not normally expected if intermediate precision is performed.

3.9.1.6 Lower limit of quantification (LOQ)

The quantitation limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be quantitatively determined with suitable precision and accuracy. The quantitation limit is a parameter of quantitative assays for low levels of compounds in sample matrices, and is used particularly for the determination of impurities and/or degradation products.

3.9.1.7 Limit of detection (LOD)

The detection limit of an individual analytical procedure is the lowest amount of analyte in a sample which can be detected but not necessarily quantitated as an exact value.

3.10 Effect of renal disease on iohexol separation

The function of the kidney is to clear nitrogenous waste products. In CKD and ESRD, these accumulated products can cause a multitude of uremic symptoms. In addition, uremic toxins are capable of causing interference in many assays. In HPLC assays, these substances may interfere by being eluted at the same time as the analyte. This could produce a confounding peak that might results in difficulty in assessing the area under the curve for the peaks of interesting compound. McCormick EM et al (32) found that ESRD samples could interfere HPLC with UV detector setting at 254 nm which is the same as iohexol detection. Therefore, ESRD samples may interfere iohexol separation. Nonetheless, the interference of the peaks of iohexol assays in uremia has not been assessed previously.

3.11 Effect of liver disease on iohexol separation

GFR studies are essential components of evaluation of subjects with advanced liver cirrhosis being considered for liver transplantation. In patients with cirrhosis, there is decreased muscle mass and malnutrition, which would lead to

underestimation of GFR when serum creatine is used to estimate GFR. As such iohexol clearance could be a very useful method to study GFR in these patients. The liver detoxifies or excretes many compounds; such compounds including bilirubin accumulate in patients with advanced liver diseases. Bilirubin is derived from hemoglobin of aged or damaged red blood cells. Elevated bilirubin found in hepatitis, hepatic duct obstructive or hemolytic anemia. Elevated bilirubin may cause interference in proportional to its concentration. Interference mechanism of bilirubin is its absorbance at 450 or 460 nm and fluorescence properties. Thus, it may interfere in colorimetric assay which measure at this wavelength. Bilirubin may interfere by participant in redox reaction, commonly found in enzymatic methods which use peroxidase and oxidase. Additionally, bilirubin may interfere in free drug assays by changing the distribution of drug between free versus complexes compartments. Interference of bilirubin to high performance liquid chromatography (HPLC) data is lack but some studies mention that high bilirubin sample can generate confounding peak in HPLC assay. Thus, interference of bilirubin may involve iohexol determination (33).

CHAPTER IV
MATERIALS AND METHODS

4.1 Standard, Reagents and Chemicals

All standard, reagents and chemicals are as following:

Table 4.1 List of standard, reagents and chemicals**.**

Standard, reagents and chemicals	Grade	Supplier
Iohexol standard	99% pure	Pharmacopoeia (USA)
Acetonitrile	HPLC	Fisher scientific (UK)
Methanol	HPLC	Fisher scientific (UK)
Orthophospharic acid	AR	Merck (USA)
Perchloric acid	AR	Acos organic (USA)

4.2 Instrumentations

Instruments and equipments used in this study are list as following:

Table 4.2 List of instruments and equipment.

Instruments	Model	Company
1. Columns	- Alltech Alltima C18 column, 5 µm, 4.6 X 250 mm	Alltech Associates (Deerfield, IL, USA)
	- Nucleosil C18 column, 5 µm, 4.6 X 250 mm	Grance Discovery Science, Deerfield, IL, USA
	- Zorbax ODS column**,** 5 µm, 4.6 X 250 mm	Agilent (Wilminton,DE,USA)

Table 4.2 (Cont.)

Instruments	Model	Company
2. Injector	Waters™ 717 plus autosampler	Waters (Milford, MA, USA)
3. Pump	Waters™ 600	Waters (Milford, MA, USA)
4. Vacuum degasser		Waters (Milford, MA, USA)
5. Detector	UV detector	Waters (Milford, MA, USA)
6. Data system	Water LC Millennium V2.10 System Manager	Waters (Milford, MA, USA)
7. Temperature controller		Waters (Milford, MA, USA)
8. Analytical balance		Sartorius (Fed.Rep, Germany)
9. pH meter	Jenway 3510	Jenway (Stone,Staffordshire ST15 0SA,UK)
10. Deionizer water system	Nanopure diamond™	Barnsted (Molsheim, France)
11. Vortex mixer		Finemixer (Seoul, Korea)
12. Auto pipettes		Biohit (Bangkok, Thailand)
13. Cylinder		Kimax (Baltimore, MD, USA)
14. Volumetric flask		Sartorius (Fed.Rep, Germany)
16. Syringe filter (0.2 μm)	PVDF (HL)	Vertical (Bangkok, Thailand)

4.3 Column specifications

Table 4.3 shows typical properties of the three columns which were used in this study.

Table 4.3 Columns specification.

Columns	Dimension (mm)	Particle size (µm)	Alkyl-bonded	End-capped	Carbon chain	Pore size (A°)
Alltech Alltima	250 : 4.6	5	polymeric	double	18	100
Nucleosil	250 : 4.6	5	monomeric	single	18	100
ZorbaxODS	250 : 4.6	5	monomeric	single	18	100

4.4 Preparation of solution and standard

4.4.1 Mobile phase

The 35 ml of acetonitrile were added and made up to 1000 ml with deionized water in volumetric flask then adjusted to pH 2.5 by addition of small amount of orthophospharic acid. The solution was filtered through 0.45 µm filter under vacuum condition and then sonicated in ultrasonic bath for 15 min.

4.4.2 Perchloric acid (5% v/v)

The 5% perchloric acid was prepared by dissolving 71.33 ml of 70% perchloric acid in distilled water to make final volume as 1000 ml in a volumetric flask. This solution was used to precipitate protein out of samples.

4.4.3 Standard iohexol solution

Stock standard iohexol were prepared by accurately dissolving 50.00 mg of pure iohexol standard in mobile phase prepared as described in section 4.4.1 and made up to 5.00 ml in volumetric flask to give concentration 10 mg/ml. This stock

solution was stored in the refrigerator at -20 ºC. Solution for experiments were prepared by serial diluted this stock solution with mobile phase to give the suitable calibration curve concentration range.

4.5 Sample extraction

EDTA plasma was used in this study. Normal samples used in method modification, optimization and validation were obtained from healthy volunteers, who were staffs in Faculty of Medicine, Ramathibodi Hospital. To assess the influence of renal and liver patient samples on iohexol separation, samples were obtained from patients who diagnosed for renal disease and liver disease by the physician, respectively.

Extraction method was modified from Soman RS et al (6) and Niculescu-Duvaz et al (7). For each sample, the 50 µl of EDTA plasma volume was precipitated with 800 µl of 5% perchloric acid in a 1.5 ml microcentrifuge tubes, then mixed for 3 min on vortex, and sonicated for 15 min. After incubation at room temperature for 30 min, a mixture was centrifuged at 3000 RPM for 10 min. The supernatant was filtered through 0.2 µm VertiPure PVDF (HL) syringe filters. The 75 µl of supernatant was injected into HPLC system with the analytical column which was previously equilibrated with mobile phase and maintained at 30 ºC.

4.6 Method modification and optimization

The method of Niculescu-Duvaz et al (7) previously described to determination of iohexol was modified and optimized. 3.5% acetonitrile in water adjusted pH to 2.5 was used as mobile phase and 300 µg/ml of iohexol were injected into HPLC to evaluate the effects of stationary phase (column) which the typical properties of the three columns were shown in Table 4.3. Performances of each column including resolution between A and B isomer, peak shape, retention time, capacity factor and selectivity were investigated and compared in this work.

4.6.1 Retention time (RT)

Retention time of exo-isomer of iohexol was obtained by injected various concentrations of standard iohexol ranged from 20 ug/ml to 640 ug/ml for 10 times and data was gave in Table 1.6 of appendix.

4.6.2 Retention factor (k′)

The retention factor (k′) is the degree of retention of the sample component in the column. Retention factor of endo- and exo-isomers of each column was calculated from equation in 3.8.3.1 and retention time of uracil was used as void time.

4.6.3 Separation factor (α)

The separation factor (α) or selectivity is a measure of relative retention k'_B/k'_A of two sample components. Selectivity of endo- and exo-isomers was calculated using equation in 3.8.3.2 where k'_B is capacity factor of exo-isomer and k'_A is capacity factor of endo-isomer, respectively.

4.6.4 Column efficiency and plate number (N)

The number of theoretical plates or plate number (N) is a measure of the efficiency of the column. Peak obtained from uracil, a standard substance for calculating column efficiency, was used to calculate N using equation in 3.8.3.3. The standard conditions and chromatogram of uracil separations for the three columns were shown in Figure 1.1, 1.2 and 1.3 in appendix, respectively.

4.6.5 Resolution (Rs)

Resolution was defined as a measure of the degree of separation of two adjacent analytes. The resolution between endo- and exo-isomers of all columns were calculated using equation in 3.8.3.4

4.6.6 Asymmetry factor (As)

The asymmetry factor (As) was used to measure the degree of peak symmetry and was defined at peak width of 10% of peak height (W). The As of endo- and exo-isomers of all columns were calculated using equation in 3.8.3.5

4.7 Validation of analytical method among the three columns

For data analysis, the peak area of exo-isomer was used to validate because it constituted more than 80% of the combined peak areas and the ratio of both isomer peaks remained constant at different iohexol concentrations under the analytical condition. Method was validated by following the International Conference on Harmonization (ICH) (31). For quantitative method, this organization recommends five parameters should be performed include:

4.7.1 Selectivity

The 100 µg/ml iohexol standard was injected to obtain retention time of A and B isomers and a total of 10 blank samples were analyzed for checking interference signal at the retention time of iohexol.

4.7.2 Evaluation of range/linearity

Stock standard iohexol was spiked into plasma to prepare samples at 6 concentrations; 20, 40, 80, 260, 320 and 640 µg/ml. The triplicate samples for each concentration were extracted as described in 4.5 and then analyzed by HPLC-UV system obtained from 4.6. The area under the curve (AUC) of B peak of each column was plotted against iohexol concentrations and regression coefficient (r^2) of each curve was evaluated.

4.7.3 Precision and accuracy/recovery

Stock standard iohexol was spiked into plasma to prepare samples at 3 concentrations; 20, 300 and 600 µg/ml. Each concentration was extracted replicate to prepare a total of 15 samples for precision study and 9 samples for accuracy study.

For precision study, % Relative standard deviations (% RSD) of the three columns were calculated as following;

$$\% \text{ RSD} = (SD/\overline{X}) \times 100$$

Where; SD = standard deviation

$\overline{X}$ = means

The recovered quantities were evaluated as % recovery for accuracy.

$$\% \text{ Recovery} = (C_{found} - C_{blank})/C_{add} \times 100$$

Where; C_{found} = measured concentration of spiked sample

C_{blank} = measured concentration of unspiked sample

C_{add} = concentration of spiked standard

4.7.4 Limit of detection (LOD) and limit of quantitation (LOQ)

Stock standard iohexol was spiked into plasma to prepare samples at lowest concentration, 20 μg/ml, and was extracted replicate to 15 samples. The limit of detection is the lowest amount analyze detectable by a procedure. The signal value of 3 and 10 times standard deviation from injected of 20 μg/ml iohexol was converted to the concentration to give the LOD and LOQ, respectively.

4.7.5 Column comparison

The comparison experiments for Alltech Alltima, Nucleosil C18 and Zorbax ODS columns based on column performance parameters including retention time, resolution between A and B isomers, peak tailing, peak high, capacity factor and selectivity of iohexol. The following constrains were imposed on some parameter; retention time should be less time, resolution between A and B isomer should be higher, peak tailing should be nearest 1 and % RSD of peak area less than 5%. Validation results were also used to consider.

4.8 Assessment of the interference of renal disease patient samples on iohexol separation

4.8.1 Renal disease samples

A total of 15 EDTA plasma samples was obtained from patients who was diagnosed for renal disease by the nephrologist. Blood urea nitrogen (BUN) and creatinine concentrations for all subjects were higher than 25 mg/dl and 2.5 mg/dl, respectively. The study was divided into two parts as following:

4.8.1.1 Interference of renal patient samples on iohexol chromatogram

Blank samples from iohexol were extracted and injected to the HPLC system. Interfering peaks on the chromatogram at the retention time of peak A and peak B were investigated.

4.8.1.2 Assessment of % recovery in renal patient samples

Three levels of standard iohexol include 20 µg/ml, 300 µg/ml and 600 µg/ml were spiked into each samples. The samples were extracted and analyzed. AUC of peak B was used to calculate % recovery. Increasing or decreasing of % recovery greater than 10% comparing to each spiked iohexol standard indicated significant influence of renal disease on iohexol determination. % Recovery was calculated as in 4.7.3.

4.9 Assessment of the interference of liver disease patient samples on iohexol separation

4.9.1 Liver disease samples

A total of 15 EDTA plasma samples was obtained from patients who was diagnosed for liver disease by the physician. Total bilirubin concentrations for all subjects ranged from 0.5 mg/dl to 27.3 mg/dl. The study was divided into two parts as following:

4.9.1.1 Interference of liver patient samples on iohexol chromatogram

Blank samples from iohexol were extracted and injected to the HPLC system. Interfering peaks on the chromatogram at the retention time of peak A and peak B were investigated.

4.9.1.2 Assessment of % recovery in liver patient samples

Three levels of standard iohexol include 20 µg/ml, 300 µg/ml and 600 µg/ml were spiked into each samples. The samples were extracted and analyzed. AUC of peak B was used to calculate % recovery. Increasing or decreasing of % recovery greater than 10% comparing to each spiked iohexol standard indicated

significant influence of liver disease on iohexol determination. % Recovery was calculated as in 4.7.3.

CHAPTER V
RESULTS

5.1 Development and optimization of analytical method for iohexol determination

The analytical method was modified from Soman et al (6) and Niculescu-Duvaz et al (7). Amount of EDTA plasma was reduced to 50 µl and 5% perchloric 800 µl. These amounts were enough for extraction and got an accurate result when injected only 75 µl into HPLC. The ratio of acetonitrile to water and pH were varied to find the optimal mobile phase. In this study, pH and ratio of acetonitrile to water could influence retention time and resolution between two isomers. Increasing concentration of acetonitrile or reducing pH resulted in reducing of retention time and resolution. After that, the optimized condition for determining iohexol in EDTA plasma was applied on the three columns; Alltech Alltima, Nucleosil C18 and Zorbax ODS.

The optimum condition of HPLC-UV to separate iohexol in EDTA plasma was summarized in Table 5.1.

Table 5.1 Optimum condition of HPLC-UV system for iohexol separation.

Parameters	**Condition**
Mobile phase	3.5 % acetonitrile in water
pH	2.5
Flow rate	1.5 ml/min
Column temperature	30 °C
Injection volume	75 µl
Detection	254 nm
Sensitivity (A.U.F.)	0.05

According to the optimum condition in Table 5.1, the three reverse-phase columns, Alltech Alltima, Nucleosil C 18 and Zorbax ODS, eluted iohexol into two isomers; endo- and exo-isomers and chromatograms of each column were shown in Figure 5.1 - 5.3, respectively.

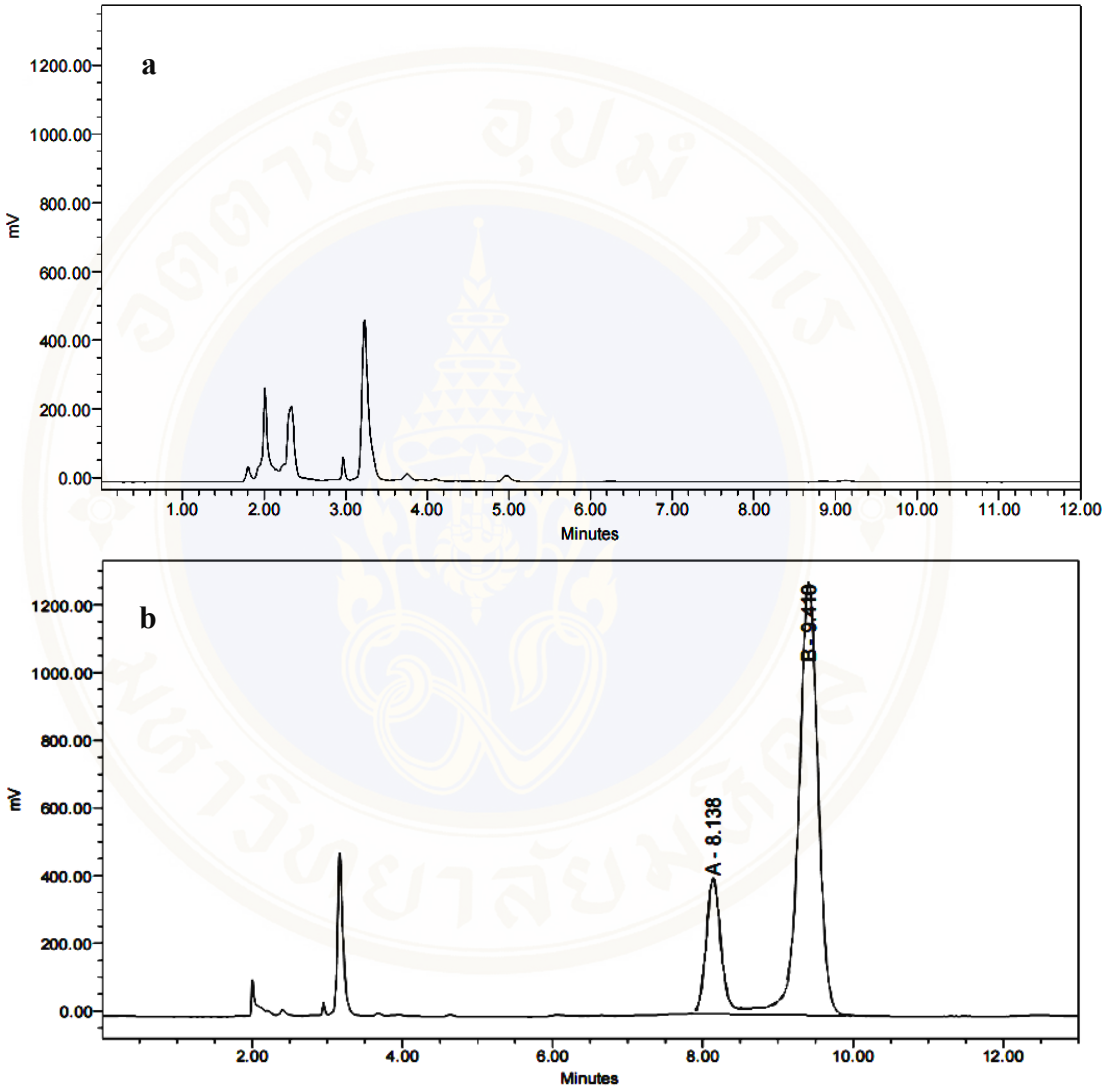

Figure 5.1 Chromatogram achieved by Alltech Alltima column using blank sample (a) and spiked 300 µg/ml iohexol sample (b).

As shown in Figure 5.1a, no interfering peak was found at the retention time of iohexol obtained from Alltech Alltima column. Endo-isomer (peak A) of iohexol eluted at 8.14 min and exo-isomer (peak B) iohexol eluted at 9.41 min, respectively (Fig. 5.1b). This chromatogram showed complete separation between endo- and exo- isomers.

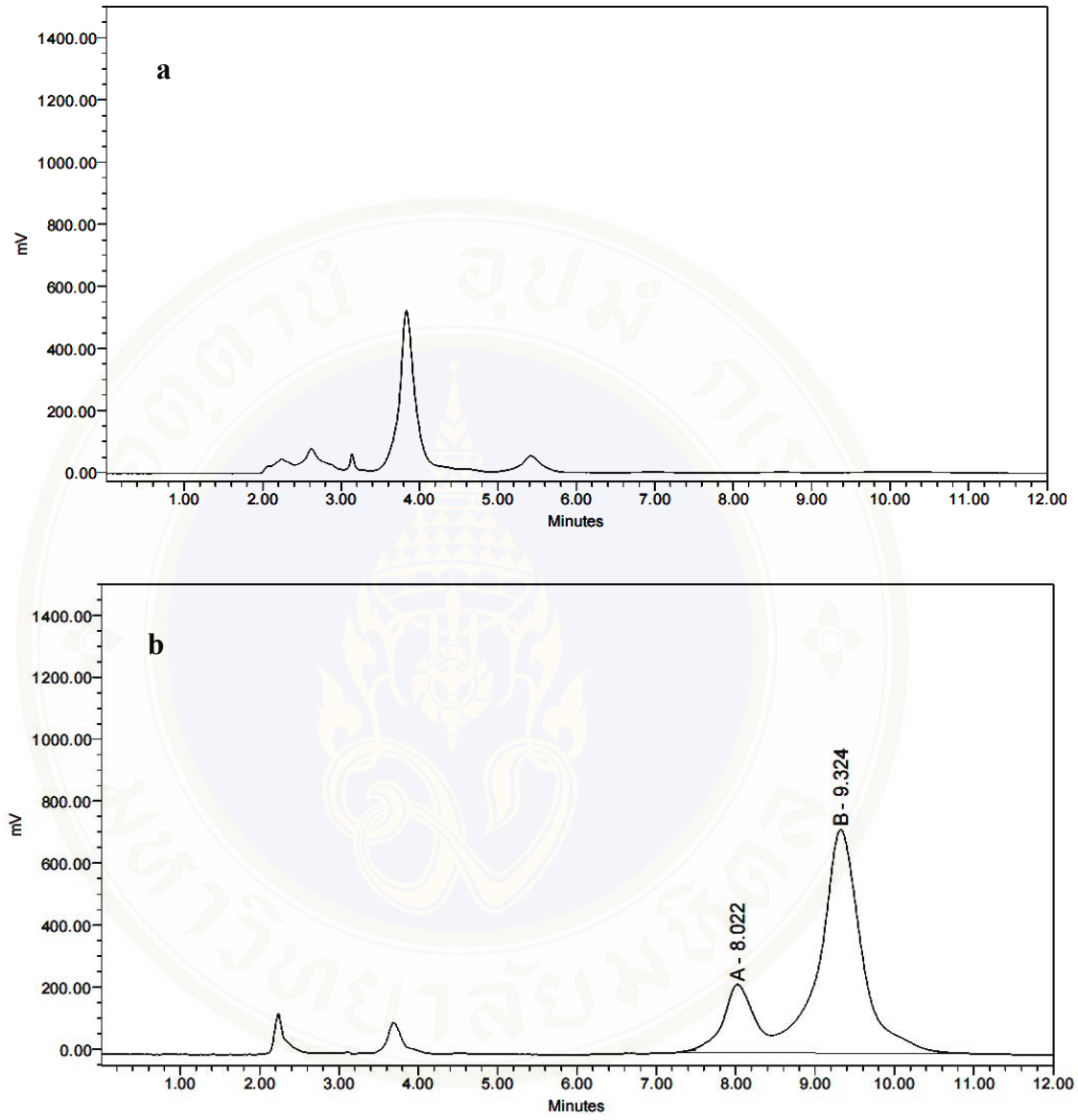

Figure 5.2 Chromatogram achieved by Nucleosil C18 column using blank sample (a) and spiked 300 µg/ml iohexol sample (b).

As shown in Figure 5.2a, no interfering peak was found at the retention time of iohexol obtained from Nucleosil C18 column. Endo-isomer (peak A) of iohexol eluted at 8.02 min and exo-isomer (peak B) iohexol eluted at 9.32 min, respectively (Fig. 5.2b). This chromatogram showed minor overlapping between endo- and exo- isomers and tailing peak was also found.

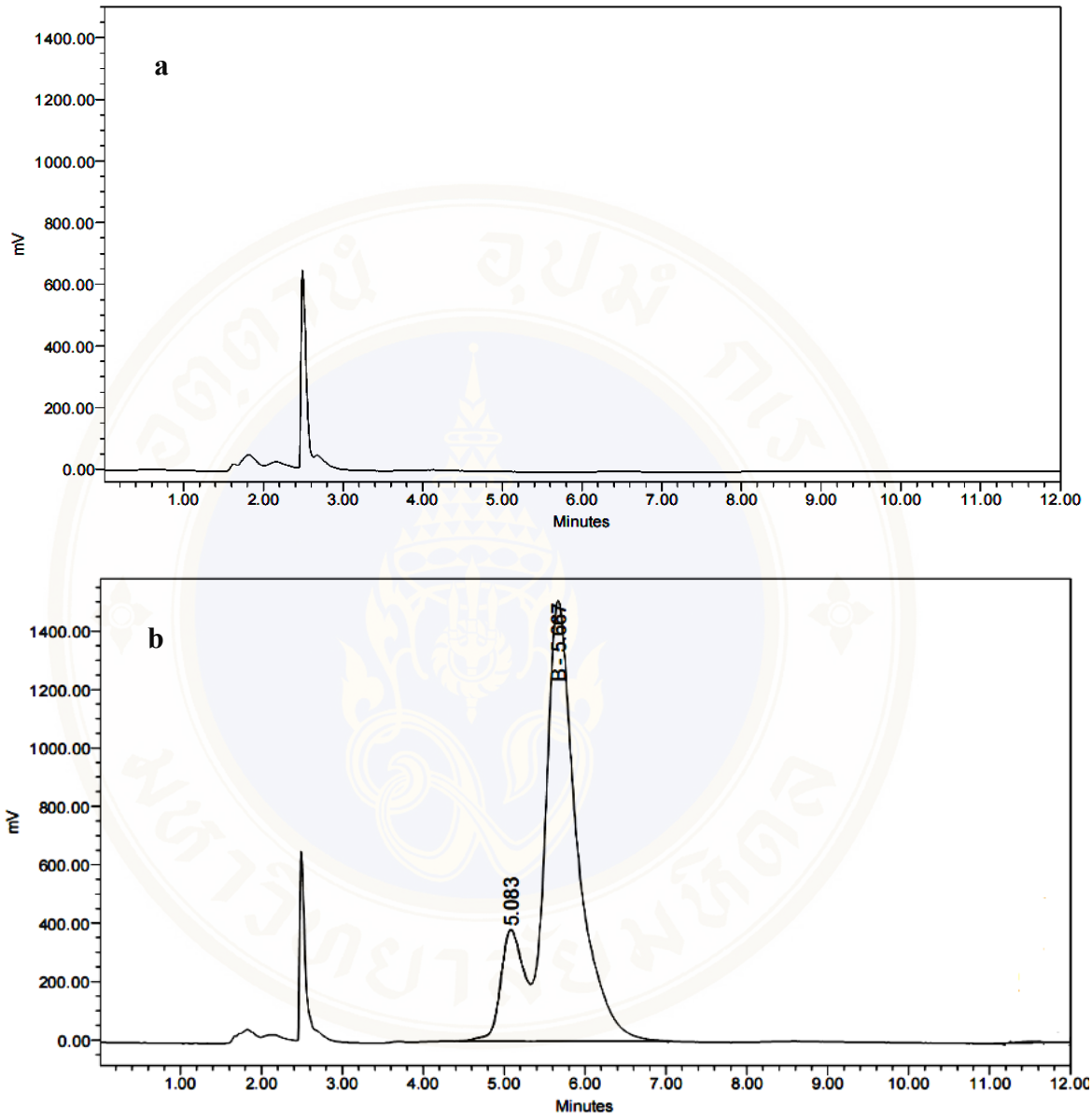

Figure 5.3 Chromatogram achieved by Zorbax ODS column using blank sample (a) and spiked 300 µg/ml iohexol sample (b).

As shown in Figure 5.3a, no interfering peak was found at the retention time of iohexol obtained from Zorbax ODS column. Endo-isomer (peak A) of iohexol eluted at 5.08 min and exo-isomer iohexol (peak B) eluted at 5.70 min, respectively (Fig. 5.3b). This chromatogram showed large overlapping between endo- and exo-isomers.

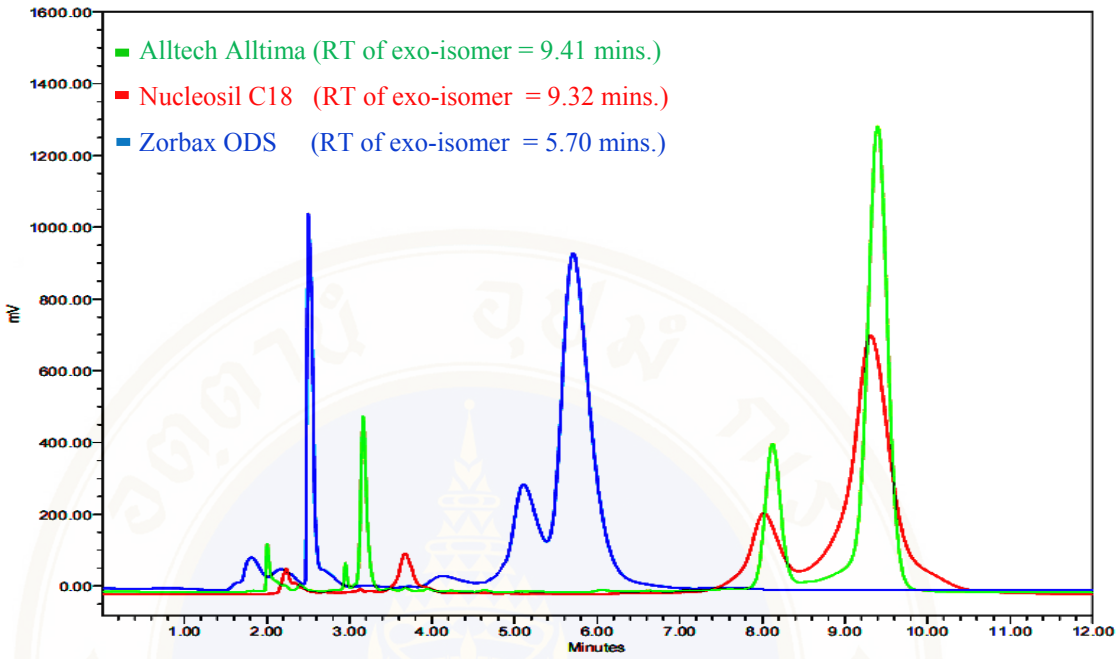

Figure 5.4 Chromatogram of 300 µg/ml iohexol using the three columns; green chromatogram represented to Alltech Alltima, red chromatogram represented to Nucleosil C18 and blue chromatogram represented to Zorbax ODS, respectively.

As shown in Figure 5.4, chromatogram achieved by Alltech Alltima obtained highest peak of exo-isomer and Nucleosil C18 obtained shortest peak of exo-isomer chromatograms. The Zorbax ODS gave the shortest retention time and Alltech Alltima gave the longest retention time. However, only chromatogram obtained from Alltech Alltima showed endo- and exo-isomers were completely separated while the others two columns showed an overlapping.

5.2 Validation of analytical method among the three columns

Exo-isomer or peak B was evaluated and validated to use in this experiment because it was a major isomer. Using EDTA plasma obtained from normal subjects, no confounding peak was found at retention time of exo-isomer for all columns.

5.2.1 Linearity of analysis range and regression analysis

Linearity of analysis range (20 - 640 µg/ml) was evaluated using triplicate samples spiked standard iohexol at level of 20, 40, 80, 160, 320 and 640 µg/ml, respectively. The regression coefficients (r^2) were acceptable for all three columns (r^2 = 0.9997 for Alltech Alltima, r^2 = 0.9995 for Nucleosil C18 and r^2 = 0.9998 for Zorbax ODS). Regression analysis of the three columns was given in Figure 5.5.

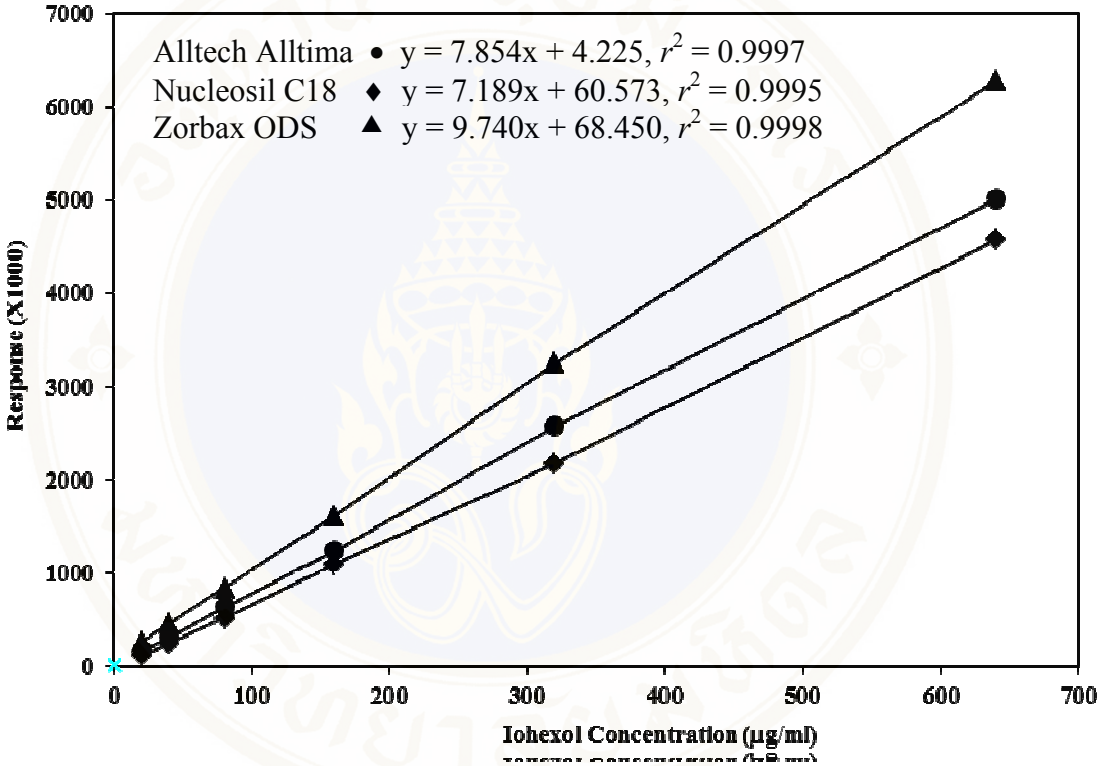

Figure 5.5 Calibration curves of Alltech Alltima (•), Nucleosil C18 (♦) and Zorbax ODS (▲) columns.

5.2.2 Precision and accuracy

The precision was expressed as relative standard deviation (% RSD) of detected concentration, acceptable range is less than 2 (30). Intra-day and inter-day precisions of Alltech Alltima at low, medium and high concentrations (20, 300 and 600 µg/ml) were lower than 2 % RSD while Nucleosil C18 and Zorbax ODS columns were higher than 2 % RSD, except intra-day precision of Zorbax ODS column at low concentration, % RSD was 1.43. Data was given in Table 5.2.

Accuracy was expressed as the % recovery and all of the three columns were in acceptable range (90% - 110%) (30). Means of % recoveries when analyzed by Alltech Alltima were 100.2 ± 2.3, 99.5 ± 0.2 and 99.9 ± 1.9 for low, medium and high iohexol concentrations, respectively. Means of % recoveries of Nucleosil C18 were 93.3 ± 2.5, 100.1 ± 1.1 and 97.9 ± 3.8 for low, medium and high concentrations, respectively. Means of % recoveries of Zorbax ODS were 97.2 ± 4.8, 99.7 ± 1.8 and 100.7 ± 1.7 for low, medium and high concentrations, respectively. Data was given in Table 5.3.

Table 5.2 Precision of iohexol determination by the three columns.

Iohexol concentrations	**% Relative Standard Deviation (% RSD)**					
	Alltech Alltima		**Nucleosil C18**		**Zorbax ODS**	
	Intra-day	**Inter-day**	**Intra-day**	**Inter-day**	**Intra-day**	**Inter-day**
20 ug/ml	1.54	1.70	4.62	5.42	1.43	2.80
300 ug/ml	1.37	1.50	2.82	4.69	2.02	3.27
600 ug/ml	0.94	1.88	3.06	2.58	2.34	2.63

Table 5.3 % Recovery of the three columns.

No.	Observed iohexol concentrations (µg/ml)								
	Alltech Alltima			Nucleosil C18			Zorbax ODS		
	Low	Medium	High	Low	Medium	High	Low	Medium	High
1	19.7	298.2	590.2	19.2	299.3	579.2	20.4	308.0	588.4
2	20.3	298.5	611.3	19.3	299.5	592.1	20.8	305.3	623.5
3	20.3	297.9	589.0	18.6	298.8	587.0	20.9	299.9	608.8
4	19.8	299.4	592.5	18.7	297.7	538.0	18.7	293.1	591.3
5	20.5	299.8	602.2	18.7	298.1	584.8	18.8	292.9	602.8
6	19.7	298.8	597.8	18.9	296.3	576.9	18.8	293.9	603.8
7	20.0	298.5	593.6	18.3	304.7	605.9	18.8	297.2	602.7
8	20.8	298.0	622.6	17.6	302.2	611.0	18.9	301.1	607.3
9	19.3	298.4	593.2	18.6	305.6	611.5	18.9	301.3	607.0
10	19.7	298.2	590.2	19.2	299.3	579.2	20.4	308.0	588.4
11	20.3	298.5	611.3	19.3	299.5	592.1	20.8	305.3	623.5
12	20.3	297.9	589.0	18.6	298.8	587.0	20.9	299.9	608.8
13	19.8	299.4	592.5	18.7	297.7	538.0	18.7	293.1	591.3
14	20.5	299.8	602.2	18.7	298.1	584.8	18.8	292.9	602.8
15	19.7	298.8	597.8	18.9	296.3	576.9	18.8	293.9	603.8
$\overline{X} \pm$ SD	20.0 ± 0.5	298.6 ± 0.6	599.2 ± 11.2	18.7 ± 0.5	300.2 ± 3.2	587.4 ± 22.7	19.4 ± 1.0	299.2 ± 5.4	604. ± 10.2
% RSD	2.3	0.2	1.9	2.7	1.1	3.9	4.9	1.8	1.7
% Recovery ± SD	100.2 ± 2.3	99.5± 0.2	99.9 ± 1.9	93.3 ± 2.5	100.1 ± 1.1	97.9 ± 3.8	97.2 ± 4.8	99.7 ± 1.8	100.7 ± 1.7

5.2.3 Limit of detection (LOD) and limit of quantitation (LOQ)

LOD of iohexol by Alltech Alltima, Nucleosil C18 and Zorbax ODS column analysis were 0.93, 2.77 and 0.86 μg/ml, respectively.

LOQ of iohexol by Alltech Alltima, Nucleosil C18 and Zorbax ODS column analysis were 3.10, 9.24 and 2.86 μg/ml, respectively.

5.2.4 Investigation of isomerization of endo- and exo-isomers

Isomerization of iohexol when analyzed by HPLC-UV technique was considered because it existed as endo- and exo-isomer peaks. Thus, selection of a major isomer peak area for quantitation was studied by calculating the percentage of endo- and exo-isomer peak areas. As summarized in Table 5.4, exo-isomer was a main isomer of iohexol and the averages of percentages of exo-isomer peak area obtained from all columns were constant at all iohexol concentrations ranged from 20 μg/ml to 640 μg/ml. The percentage of endo-isomer peak area is about 20% and the percentage of exo-isomer peak area is about 80%. Therefore, the exo-isomer peak area was used for calibration curve and all iohexol concentration calculations in this study.

Table 5.4 Averages of percentages of exo-isomer iohexol peak area obtained from the three columns at various iohexol concentrations.

Concentrations of iohexol	Percentages of exo-isomer iohexol peak area		
	$\bar{x}$	±	SD
20 μg/ml	82.53	±	1.98
40 μg/ml	81.50	±	0.49
80 μg/ml	81.10	±	0.63
160 μg/ml	81.22	±	0.68
320 μg/ml	80.98	±	0.77
640 μg/ml	81.08	±	0.99

5.3 Performance of the three columns

As guided in the selection of HPLC column, the good resolution of iohexol isomers and good peak shape within a short time as possible are required. Although Alltech Alltima used the longest time to elute iohexol, this column yielded a clear separation and good peak shape. The chromatograms achieved by Nucleosil C18 and Zorbax ODS columns showed overlapping between endo- and exo-isomer peaks. Nevertheless, Zorbax ODS used shortest time to elute iohexol but it got poorest resolution. General performances of the three columns using standard uracil, phenol, N,N- diethyl-m-toluamide and toluene for test were shown in Table C1-3 and Figure C1-3, respectively (appendix C). For standard iohexol separation, performances of the three columns were shown in Table 5.5 including separation time (RT) of peak A and B, % RSD of RT, capacity factor (k'), asymmetry factor (As), resolution (Rs), and selectivity (α) respectively. Alltech Alltima column showed a good performance because all parameters were in the acceptance criterions while other columns were not. Only Zorbax ODS gave capacity factor value less than 2. Resolutions for Nucleosil and Zorbax ODS were less than 1.2. This data indicated that iohexol isomers could not be completely separated by these two columns as shown in Figure 5.2b and 5.3b, respectively.

Table 5.5 Performance of the three columns.

Columns	Parameters (acceptance criterions)								
	Separation time		% RSD of RT	k'_1 (>2)	k'_2 (>2)	As_1 (0.8-1.2)	As_2 (0.8-1.2)	Rs (>1.2)	α (>1)
	peak A	peak B							
Alltech Alltima	8.14	9.41	0.30	2.32	3.18	1.00	1.00	1.50	1.24
Nucleosil C18	8.02	9.32	0.44	1.85	2.47	1.16	0.80	0.90	1.24
Zorbax ODS	5.08	5.70	0.94	0.68	1.33	1.00	1.00	0.70	1.20

% RSD is % Relative Standard Deviation of retention time of exo-isomer, Rs is resolution between endo- and exo-iosmers, k'_1 is capacity factor of endo-isoform, k'_2 is capacity factor of exo-isoform, As_1 is asymmetry factor of endo-isomer, As_2 is asymmetry of exo-isomer and α is selectivity, respectively.

The performance of an individual column based on several parameters such as retention time, capacity factor, asymmetric factor, resolution and selectivity was considered in this study as recommended by the ICH (30, 34). Alltech Alltima gave the best chromatogram characteristic, clearest separation between endo- and exo-isomers of iohexol and all performance parameters were in the acceptance criterions; however, it used longest time to elute iohexol. Nucleosil C18 and Zorbax ODS were similar properties such as single end-cap and monomericallic silane bonded phase. Although the performances of both columns were similar, chromatogram obtained from Nucleosil C18 was less overlapping than Zorbax ODS. Because Niculescu-Duvaz (7) used Nucleosil C18 for iohexol determination, this study selected Nucleosil C18 column to represent a single end-cap and monomericallic silane bonded phase and as a reference column to investigate the effects of renal and liver diseases on iohexol separation.

5.4 Assessment of the interference of renal disease patient samples on iohexol separation

5.4.1 Interference of renal patient samples on iohexol chromatogram

Interfering peaks at the retention time of iohexol were investigated by using 15 blank samples obtained from renal patients. Interfering peaks were not observed at the retention time of exo-isomer of iohexol but were observed at the retention time of endo-isomer in some samples. Chromatograms were shown in Figure 5.6. Using renal patient plasma, the chromatogram showed a high interfering peak at retention time of endo-isomer even no spiked iohexol (green line). This phenomenon was found in both chromatograms obtained from Alltech Alltima (Fig. 5.6a) and Nucleosil (Fig. 5.6b). However, when overlaid this chromatogram with a chromatogram of 300 μg/ml iohexol spiked in normal plasma (blue line), this interfering peak was not overlapping to exo-isoform achieved by Alltech Alltima while it was overlapping to exo-isoform achieved by Nucleosil column.

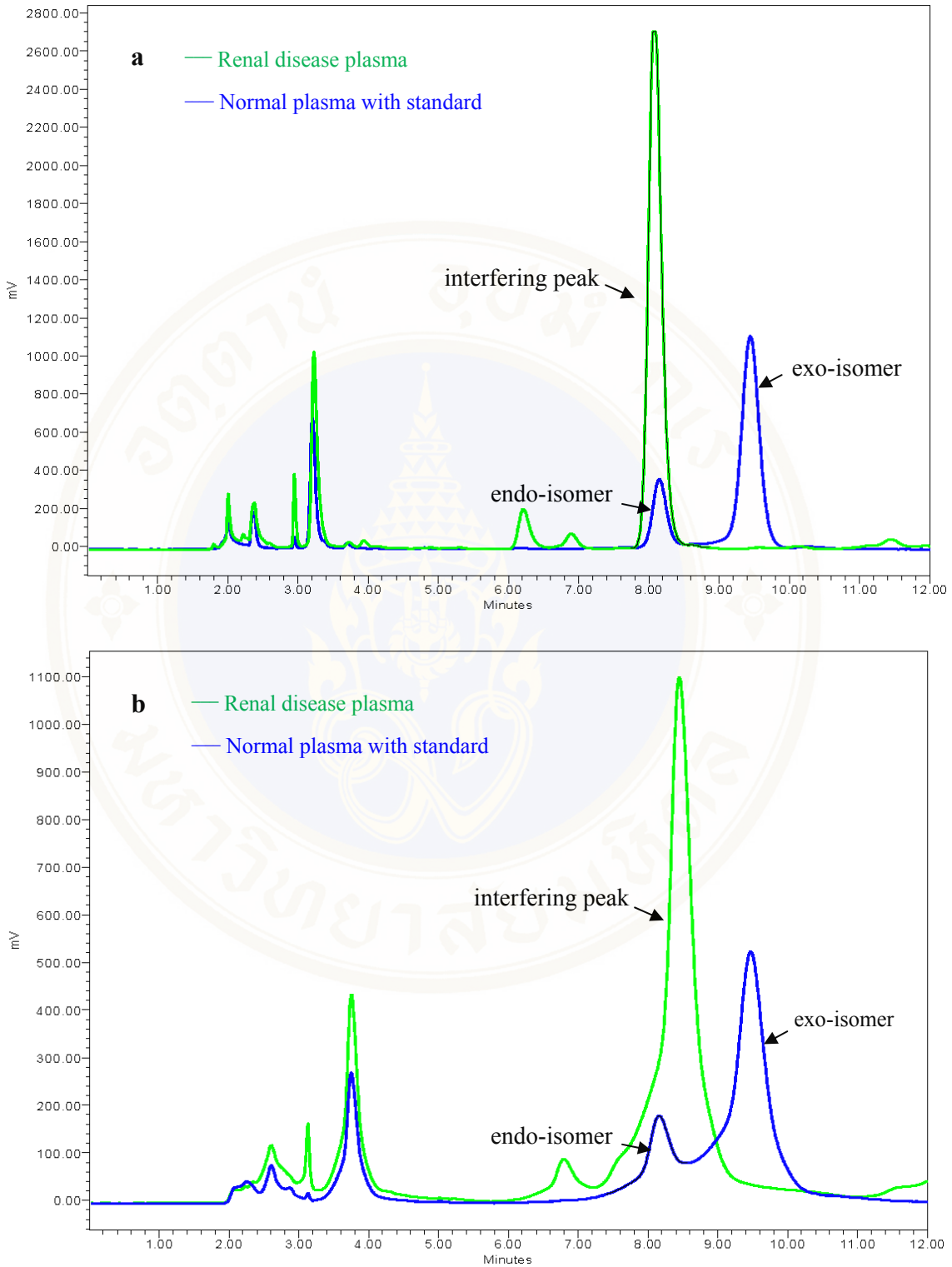

Figure 5.6 Representative of overlaid chromatograms between renal patient plasma (green line) and healthy person plasma with spiked 300 µg/ml iohexol concentration (blue line) from Alltech Alltima (a) and Nucleosil C18 (b) columns, respectively.

5.4.2 Assessment of % recovery in renal patient plasma

To investigate interfering peak from renal patient plasma which might affected accuracy of method, % recovery study of iohexol concentration was performed as described in 4.8.1.2 (n=15). The % recoveries and standard deviations of Alltech Alltima column at low, medium and high concentrations of iohexol in renal plasma were 101.5 ± 8.5, 100.2 ± 2.9 and 101.6 ± 2.4, respectively. For Nucleosil C18, they were 107.8 ± 28.8, 97.6 ± 6.1 and 100.3 ± 3.8, respectively. Considering % recovery of an individual patient in each concentration, the results showed Nucleosil C18 gave % recovery greater than the ICH criteria (30) as shown in Table 5.6. The graphical data as shown in Figure 5.7a clearly indicated that most of % recoveries at low iohexol concentration obtained from Nucleosil C18 lay out the acceptable criteria (90% - 110%), especially in sample no. 3, 9, 10 and 14 (125.5%, 183.5%, 141.5% and 131.0%, respectively). For Alltech Alltima, only sample no. 3, no. 9 and no. 11 were not in the control limit criteria but it gave better accuracy than Nucleosil C18 (118%, 119.5% and 84.0, respectively). Although % recoveries of both columns were accepted at medium (Fig. 5.7b) and high (Fig. 5.7c) iohexol concentrations, Alltech Alltima revealed better accuracy and no data were out of criterion. The raw data of observed iohexol concentrations which used to calculate % recovery were shown in Table D1 (appendix D). % Relative standard deviation (% RSD) was high at low concentration for both columns; however, Alltech Alltima gave smaller value than another at all three concentrations. The differences of iohexol concentration at low, medium and high levels between the two columns were also compared using paired *t*-test and the significant level was sat at *p*-value 0.05. There was no significant difference of iohexol concentration separated by both columns at all levels.

Table 5.6 % Recovery study for exo-isomer in renal patient plasma.

No.	% Recovery					
	Alltech Alltima			Nucleosil C18		
	Low	Medium	High	Low	Medium	High
1	103.0	101.7	97.1	116.0	101.0	100.6
2	102.5	99.5	98.7	84.5	94.6	103.5
3	118.0	100.1	103.3	125.5	92.4	100.0
4	106.0	97.0	98.6	83.0	98.6	97.9
5	97.0	98.0	100.3	81.0	99.9	107.1
6	95.0	97.9	100.7	109.0	103.9	104.9
7	97.5	95.5	101.1	89.5	85.3	100.0
8	100.5	100.2	99.6	82.0	105.8	97.6
9	119.5	97.7	102.5	183.5	95.6	97.7
10	100.0	101.5	104.4	141.5	89.2	91.5
11	84.0	106.5	104.5	91.0	95.8	100.2
12	100.0	99.6	104.0	99.5	94.5	100.1
13	101.0	101.2	103.2	85.0	97.0	103.6
14	98.5	103.8	101.4	131.0	104.7	98.1
15	100.5	102.9	104.5	114.5	106.1	102.0
% Recovery ± SD	101.5 ± 8.5	100.2 ± 2.9	101.6 ± 2.4	107.8 ± 28.8	97.6 ± 6.1	100.3 ± 3.8

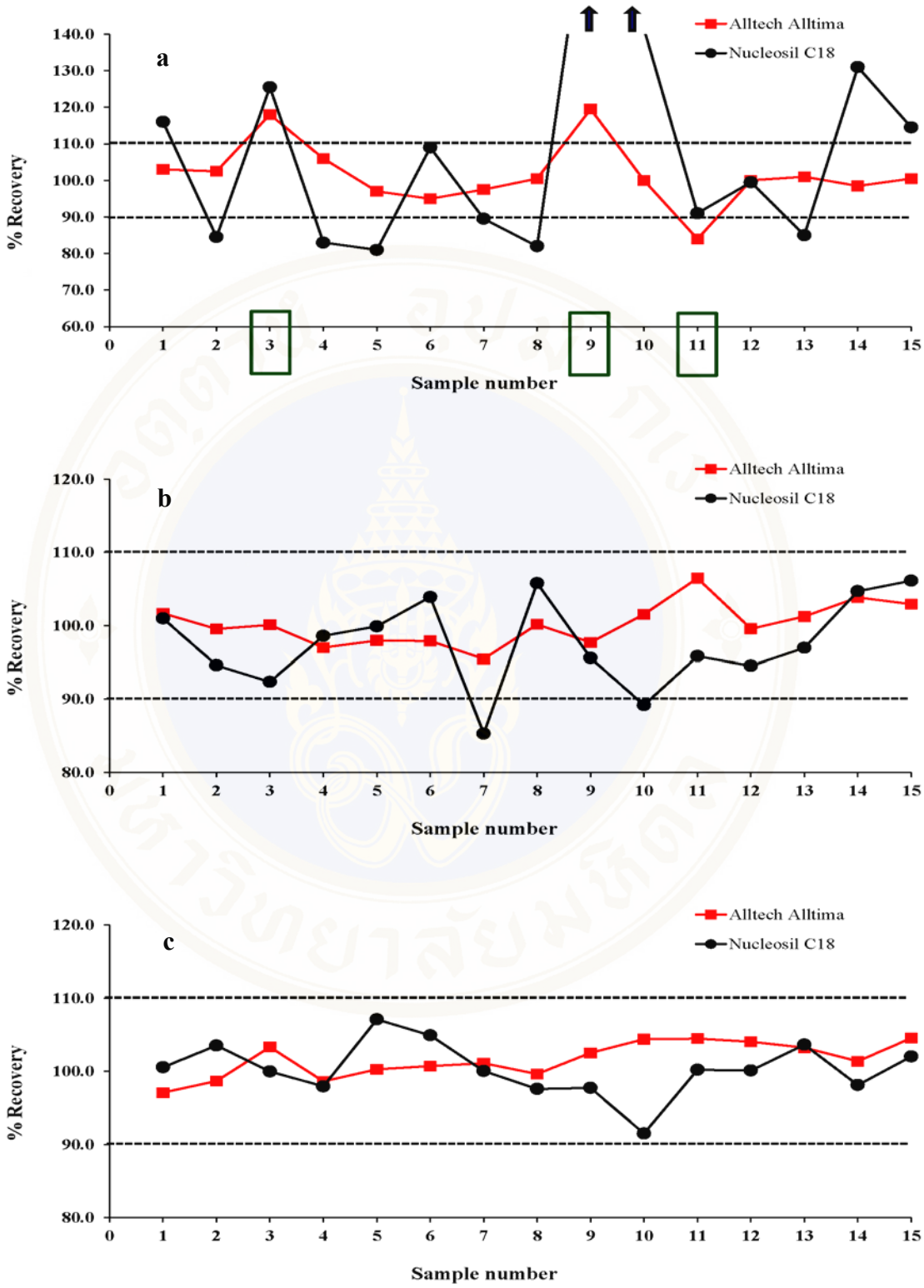

Figure 5.7 The graphical data of % recovery of an individual patient at low (a), medium (b) and high (c) iohexol concentrations achieved by Alltech Alltima (red) and Nucleosil C18 (black) columns.

Since the most of results from renal patient plasma showed interference at endo-isomer peak, the ratio of endo- and exo-isomers peak area of iohexol were concerned whether they have any effect on the calculation of iohexol concentration. This study hypothesized that interference substances in renal patient plasma might contributed to change this ratio leading to the error on data integration. To investigate this circumstance, percentages of endo- and exo-isomers peak area of iohexol and % recovery between observed and expected iohexol at low, medium, and high concentrations were calculated as showed in Table 5.7. The result showed that although the ratio of endo- and exo-isomer peak area from Alltech Alltima were changed by the interferences, there is no absolute error appeared in % recovery of iohexol concentration. For example, the percentages of endo- and exo-isomers peak area in sample no. 3, 7, 10, 12 and 15 were not constant as found in others samples, but % recovery obtained in these samples were within 100 ± 10%, except sample no. 3. The reason of this exception is the chromatogram of this sample (Fig. 5.8a) showed interfering peak at RT of both endo- and exo-isomers when overlaid chromatograms between its blank (green line) and spiked of standard iohexol (blue line) samples. Moreover, % recovery of other two samples (no. 9 and no. 11) were greater than 10% at the low concentration even theirs ratio were constant. In these samples, the interfering peak appeared at the same RT of peak B was found as sample no. 3 (Fig. D1 and D2 in appendix D, respectively). For Nucleosil C18, most of % recovery at the low concentration were greater than 10% even though the ratio were constant or not. Considering chromatograms of these samples, interfering peak at both RT of endo-isomer and overlapping at exo-isomer were found (Fig. 5.8b). At the medium and high iohexol concentrations, most of % recovery was lower than 10%. The data showed that Alltech Alltima was less affected from renal disease patients than those Nucleosil C18 and poor iohexol separation leading to the worst data integration which resulted in large bias.

Table 5.7 Summarized percentages of endo- and exo-isomers of iohexol and % recovery in renal disease.

No	Alltech Alltima									Nucleosil								
	20 µg/ml			300 µg/ml			600 µg/ml			20 µg/ml			300 µg/ml			600 µg/ml		
	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery
1	17.1	82.9	103.0	18.3	81.7	101.7	19.8	80.2	97.1	19.8	80.2	116.0	16.8	83.2	101.0	17.9	82.1	100.6
2	18.2	81.8	102.5	19.2	80.8	99.5	19.2	80.8	98.7	20.1	79.9	84.5	16.5	83.5	94.6	17.6	82.4	103.5
3	80.3	19.7	118.0	40.9	59.1	100.1	30.7	69.3	103.3	81.6	18.4	125.5	20.2	79.8	92.4	19.0	81.0	100.0
4	16.0	84.0	106.0	19.1	80.9	97.0	19.6	80.4	98.6	23.2	76.8	83.0	19.5	80.5	98.6	18.2	81.8	97.9
5	17.7	82.3	97.0	18.9	81.1	98.0	19.1	80.9	100.3	28.1	71.9	81.0	18.0	82.0	99.9	17.3	82.7	107.1
6	20.6	79.4	95.0	19.1	80.9	97.9	20.2	79.8	100.7	19.5	80.5	109.0	16.4	83.6	103.9	18.7	81.3	104.9
7	84.0	16.0	97.5	45.3	54.7	95.5	32.9	67.1	101.1	82.3	17.7	89.5	43.7	56.3	85.3	18.9	81.1	100.0
8	30.0	70.0	100.5	18.4	81.6	100.2	19.9	80.1	99.6	22.6	77.4	82.0	16.5	83.5	105.8	17.7	82.3	97.6
9	18.8	81.2	119.5	18.1	81.9	97.7	18.8	81.2	102.5	21.5	78.5	183.5	17.2	82.8	95.6	18.3	81.7	97.7
10	94.5	5.5	100.0	65.5	34.5	101.5	51.2	48.8	104.4	96.9	3.1	141.5	69.9	30.1	89.2	55.7	44.3	91.5
11	16.2	83.8	84.0	20.2	79.8	106.5	20.4	79.6	104.5	33.0	67.0	91.0	18.9	81.1	95.8	19.4	80.6	100.2
12	51.1	49.9	100.0	25.4	74.6	99.6	24.2	75.8	104.0	72.6	27.4	99.5	28.8	71.2	94.5	24.6	75.4	100.1
13	21.9	78.1	101.0	19.4	80.6	101.2	20.1	79.9	103.2	25.4	74.6	85.0	18.3	81.7	97.0	20.5	79.5	103.6
14	26.3	73.7	98.5	20.1	79.9	103.8	19.7	80.3	101.4	18.7	81.3	131.0	18.3	81.7	104.7	19.7	80.3	98.1
15	45.4	54.6	100.5	25.0	75.0	102.9	22.6	77.4	104.5	69.9	30.1	114.5	25.6	74.4	106.1	22.6	77.4	102.0

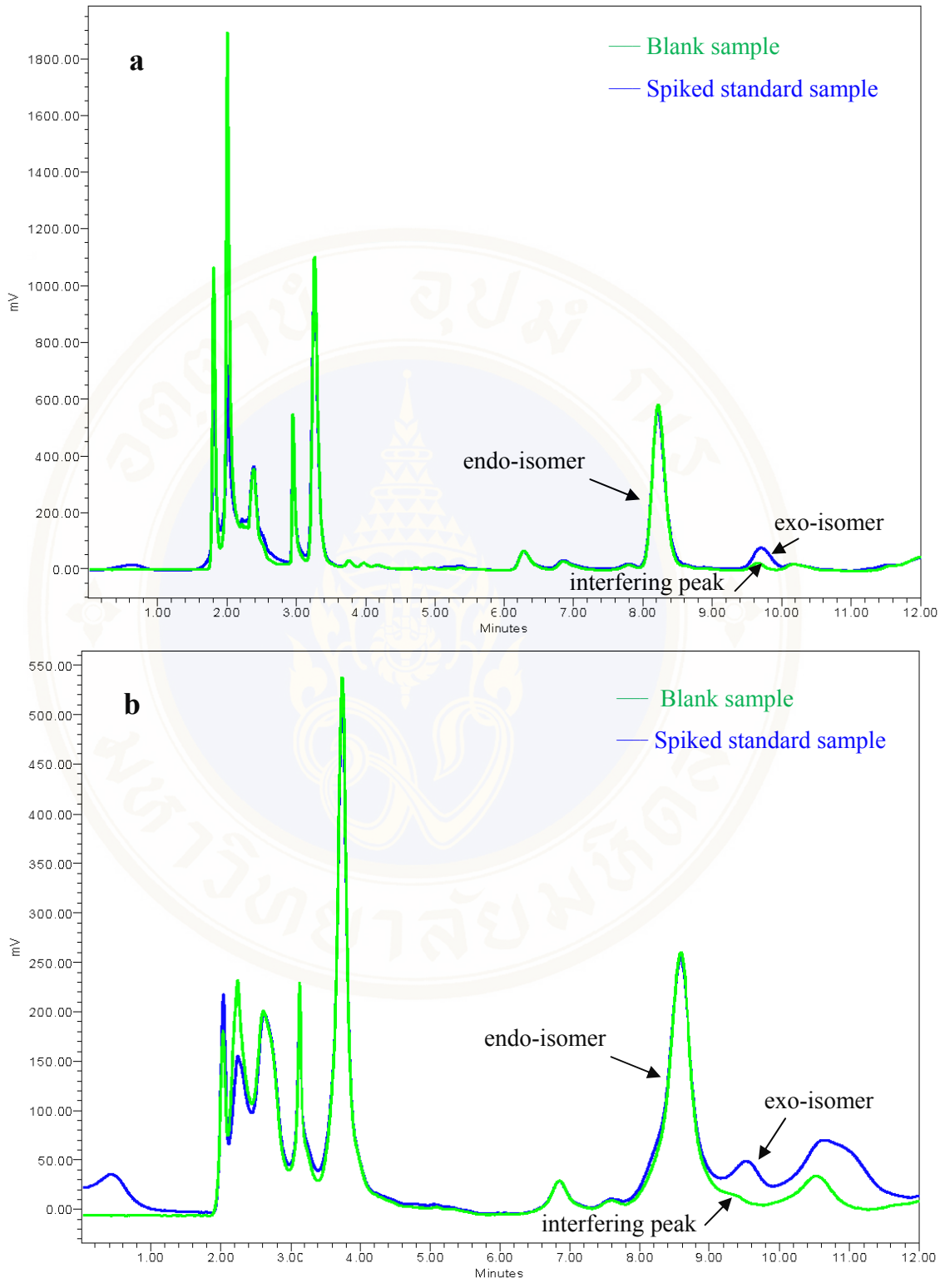

Figure 5.8 Chromatogram of renal disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 3) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

5.5 Assessment of the suitable of iohexol separation method in liver disease patients

5.5.1 Interference of liver patient samples on iohexol chromatogram

In liver patient plasma, the chromatogram (Fig. 5.9) showed a small interfering peak at retention time of endo-isomer for Alltech Alltima (Fig. 5.9a) and between retention time of endo- and exo-isomers for Nucleosil (Fig. 5.9b) even using sample without iohexol (green line). Similar to overlay chromatogram of renal patient plasma, when overlaid this chromatogram with a chromatogram of 300 μg/ml iohexol spiked in normal plasma (red line), this interfering peak was not overlapping to exo-isoform for Alltech Alltima but showed overlapping at endo- and exo-isomers for Nucleosil C18.

5.5.2 Assessment of % recovery in liver patient plasma

To investigate effect of interfering peak from liver patient plasma on accuracy of method, % recovery study of exo-isomer was performed. The % recoveries and standard deviations of Alltech Alltima column at low, medium and high concentrations of iohexol in liver plasma were 99.1 ± 5.0, 102.9 ± 3.1 and 99.2 ± 2.0, respectively. For Nucleosil C18, they were 101.5 ± 13.4, 103.3 ± 3.2 and 101.5 ± 3.5, respectively. Considering % recovery of an individual patient in each concentration, the results showed Nucleosil C18 gave % recovery greater than the ICH criteria (30) as shown in Table 5.8. The graphical data as shown in Figure 5.10a clearly indicated that % recoveries of samples no. 3, 6, 7, 9, 12 and 15 (76.0%, 114.0%, 112..5%, 123.5%, 121.5% and 81.0%, respectively) at low iohexol concentration obtained from Nucleosil C18 lay out the acceptable criteria (90% - 110%), especially in sample no. 3, 9, 12 and 15. Although % recoveries of Nucleosil were accepted at both medium (Fig. 5.10b) and high (Fig. 5.10c) iohexol concentrations, Alltech Alltima revealed better accuracy and no data were out the control limit at all iohexol concentrations. The raw data of observed iohexol concentrations which used to calculate % recovery were shown in Table D2 (appendix D). % Relative standard deviation (% RSD) was high at low concentration for both columns; however, Alltech Alltima gave smaller

value than another at all three concentrations. The differences of iohexol concentration at low, medium and high levels between the two columns were also compared using paired *t*-test and the significant level was sat at *p*-value 0.05. There was no significant difference of iohexol concentration separated by both columns at all levels. In chromatogram achieved by Alltech Alltima (Fig. 5.11a), interfering peak was found at only the retention time of endo-isomer and no overlapping to exo-isomer. However, when considered at chromatogram of the same sample (sample no. 9) from Nucleosil C18 (Fig. 5.11b), interfering peak at the retention of endo-isomer and overlapping between this peak and exo-isomer were found. Thus, this data indicated that liver disease affected on iohexol separation and data integration by Nucleosil C18, not Alltech Alltima.

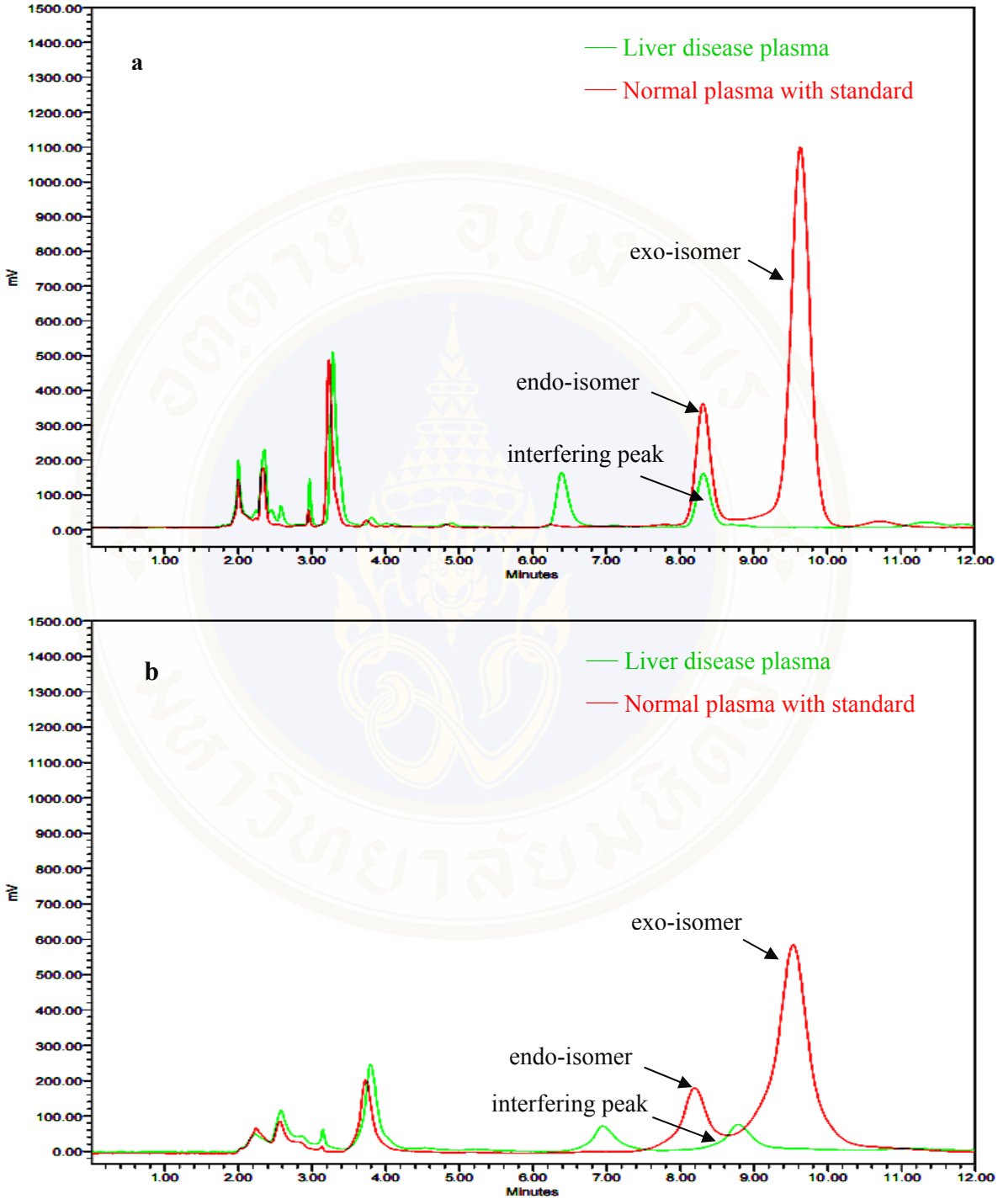

Figure 5.9 Representative of overlaid chromatograms between liver patient plasma (green line) and healthy person plasma with spiked 300 µg/ml iohexol concentration (red line) from Alltech Alltima (a) and Nucleosil C18 (b) columns, respectively.

Table 5.8 % Recovery study for exo-isomer in liver patient plasma.

No.	Total bilirubin (mg/dl)	% Recovery					
		Alltech Alltima			Nucleosil C18		
		Low	Medium	High	Low	Medium	High
1	0.5	104.5	106.8	103.0	103.5	106.7	103.0
2	0.6	97.0	108.6	97.5	92.0	108.2	97.2
3	0.8	97.5	105.2	103.5	76.0	104.1	103.8
4	0.9	101.0	100.0	99.4	97.5	99.2	102.7
5	1.0	96.0	100.3	97.8	92.5	100.4	96.4
6	1.0	91.0	102.4	97.1	114.0	100.8	100.1
7	1.2	99.0	103.1	100.2	112.5	102.2	103.8
8	1.4	103.0	102.2	97.3	96.0	101.9	97.0
9	2.5	105.0	102.4	98.6	123.5	99.3	100.9
10	3.6	108.5	100.4	98.8	100.5	106.6	105.5
11	9.7	90.5	100.2	100.1	104.5	104.1	103.2
12	10.7	96.5	104.0	99.9	121.5	105.9	105.6
13	20.5	102.5	97.4	98.8	108.0	101.8	105.8
14	23.7	98.5	102.4	100.2	99.0	100.4	102.0
15	27.3	96.0	107.7	96.6	81.0	108.4	95.8
% Recovery ± SD		99.1±5.0	102.9±3.1	99.2±2.0	101.5±13.4	103.3±3.2	101.5±3.5

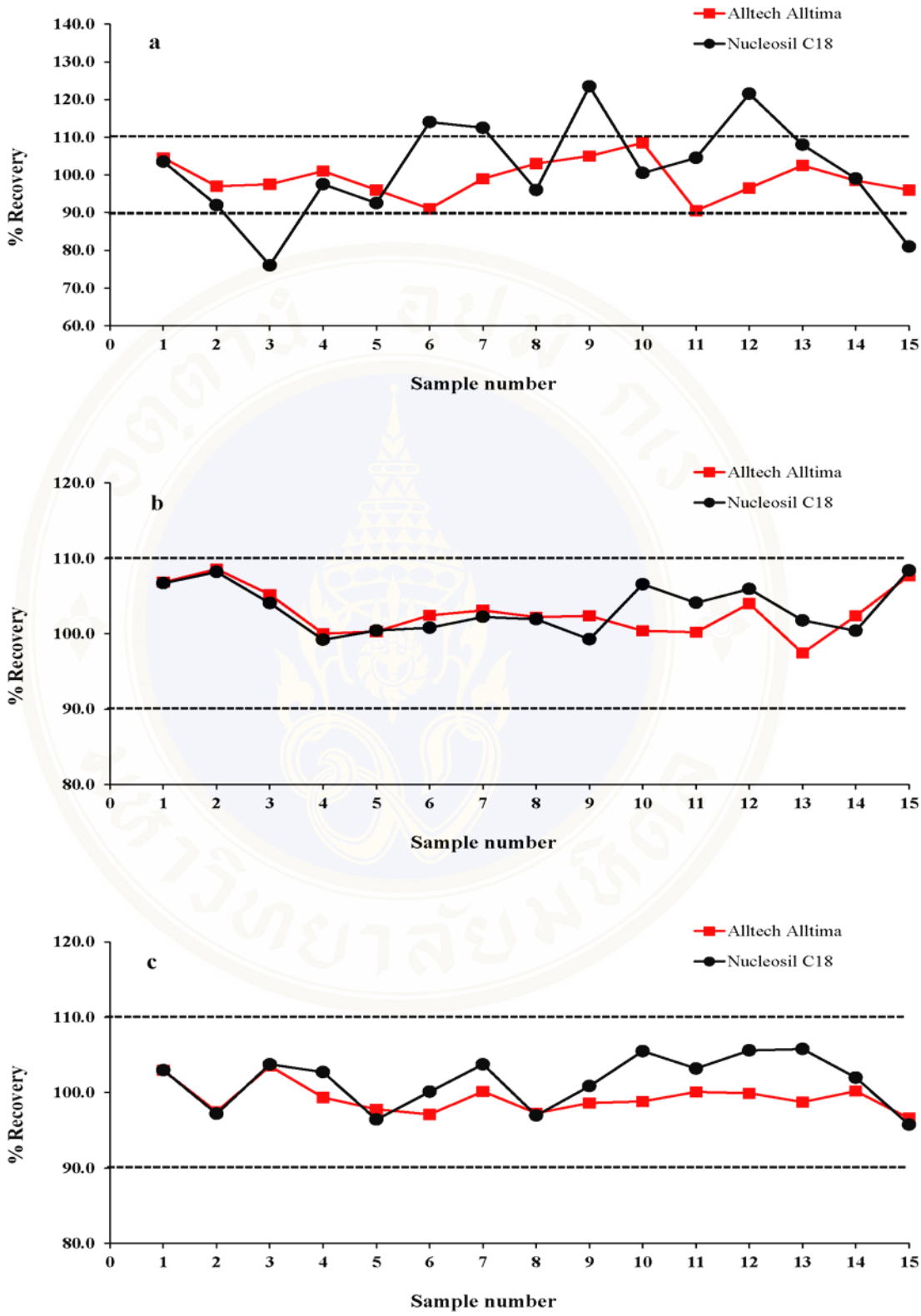

Figure 5.10 The graphical data of % recovery of an individual patient at low (a), medium (b) and high (c) iohexol concentrations achieved by Alltech Alltima (red) and Nucleosil C18 (black) columns.

Since some results from liver patient plasma showed interference at endo-isomer peak, the ratio of endo- and exo-isomers peak area of iohexol were concerned whether they have any effect on the calculation of iohexol concentration. This study hypothesized that interference substances in liver patient plasma might contributed to change this ratio leading to the error on data integration. To investigate this circumstance, percentages of endo- and exo-isomers peak area of iohexol and % recovery between observed and expected iohexol at low, medium, and high concentrations were calculated as showed in Table 5.9. The result showed that only the percentages of endo- and exo-isomers peak area in sample no. 9 was not constant as found in others samples, however % recovery obtained by Alltech Alltima in this samples were less than 10% at all iohexol concentrations. Although the ratio of endo- and exo-isomer peak area from Alltech Alltima was changed by the interferences, there is no absolute error appeared in % recovery of iohexol concentration. The chromatogram of this sample achieved by Alltech Alltima was shown in Figure 5.11a. For Nucleosil C18, some % recovery at the low concentration was greater than 10% even though the ratio were constant or not. Considering chromatogram of the same sample, interfering peak at both RT of endo-isomer and overlapping at exo-isomer were found (Fig. 5.11b). At the medium and high iohexol concentrations, most of % recovery was accepted. The data showed that Alltech Alltima was not affected from liver disease patients while Nucleosil C18 was affected, especially in the low concentrations. Poor iohexol separation achieved by Nucleosil C18 leaded to the worst data integration which resulted in large bias.

Table 5.9 Summarized percentages of endo- and exo-isomers of iohexol and % recovery in liver disease.

No	Total bilirubin (mg/dl)	Alltech Alltima									Nucleosil								
		20 µg/ml			300 µg/ml			600 µg/ml			20 µg/ml			300 µg/ml			600 µg/ml		
		A	B	% Recovery	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery	A	B	% Recovery
1	0.5	17.4	82.6	104.5	19.6	80.4	106.8	19.7	80.3	103.0	11.5	88.5	103.5	19.1	80.9	106.7	19.5	80.5	103.0
2	0.6	17.0	83.0	97.0	19.5	80.5	108.6	19.8	80.2	97.5	11.0	89.0	92.0	19.2	80.8	108.2	19.5	80.5	97.2
3	0.8	18.2	81.8	97.5	19.6	80.4	105.2	19.7	80.3	103.5	11.9	88.1	76.0	19.2	80.8	104.1	19.7	80.3	103.8
4	0.9	18.3	81.7	101.0	17.8	82.2	100.0	18.9	81.1	99.4	18.0	82.0	97.5	17.6	82.4	99.2	17.1	82.9	102.7
5	1.0	17.8	82.2	96.0	20.2	79.8	100.3	20.0	80.0	97.8	13.8	86.2	92.5	19.4	80.6	100.4	19.0	81.0	96.4
6	1.0	18.6	81.4	91.0	19.7	80.3	102.4	19.8	80.2	97.1	14.4	85.6	114.0	19.5	80.5	100.8	19.6	80.4	100.1
7	1.2	18.6	81.4	99.0	19.6	80.4	103.1	19.0	81.0	100.2	13.0	87.0	112.5	19.5	80.5	102.2	13.8	86.2	103.8
8	1.4	17.1	82.9	103.0	19.7	80.3	102.2	19.8	80.2	97.3	12.2	87.8	96.0	19.0	81.0	101.9	19.4	80.6	97.0
9	2.5	60.1	39.9	105.0	25.5	74.5	102.4	22.1	77.9	98.6	62.2	37.8	123.5	25.5	74.5	99.3	22.8	77.2	100.9
10	3.6	17.6	82.4	108.5	27.1	72.9	100.4	19.4	80.6	98.8	27.0	73.0	100.5	18.6	81.4	106.6	19.2	80.8	105.5
11	9.7	23.4	76.6	90.5	20.8	79.2	100.2	19.7	80.3	100.1	15.4	84.6	104.5	19.0	81.0	104.1	19.4	80.6	103.2
12	10.7	14.9	85.1	96.5	20.6	79.4	104.0	20.3	79.7	99.9	20.7	79.3	121.5	19.5	80.5	105.9	19.3	80.7	105.6
13	20.5	12.6	87.4	102.5	19.2	80.8	97.4	19.0	81.0	98.8	18.0	82.0	108.0	19.2	80.8	101.8	19.3	80.7	105.8
14	23.7	13.8	86.2	98.5	20.3	79.7	102.4	19.9	80.1	100.2	15.5	84.5	99.0	19.1	80.9	100.4	19.3	80.7	102.0
15	27.3	18.2	81.8	96.0	22.0	78.0	107.7	20.8	79.2	96.6	35.7	64.3	81.0	20.2	79.8	108.4	19.8	80.2	95.8

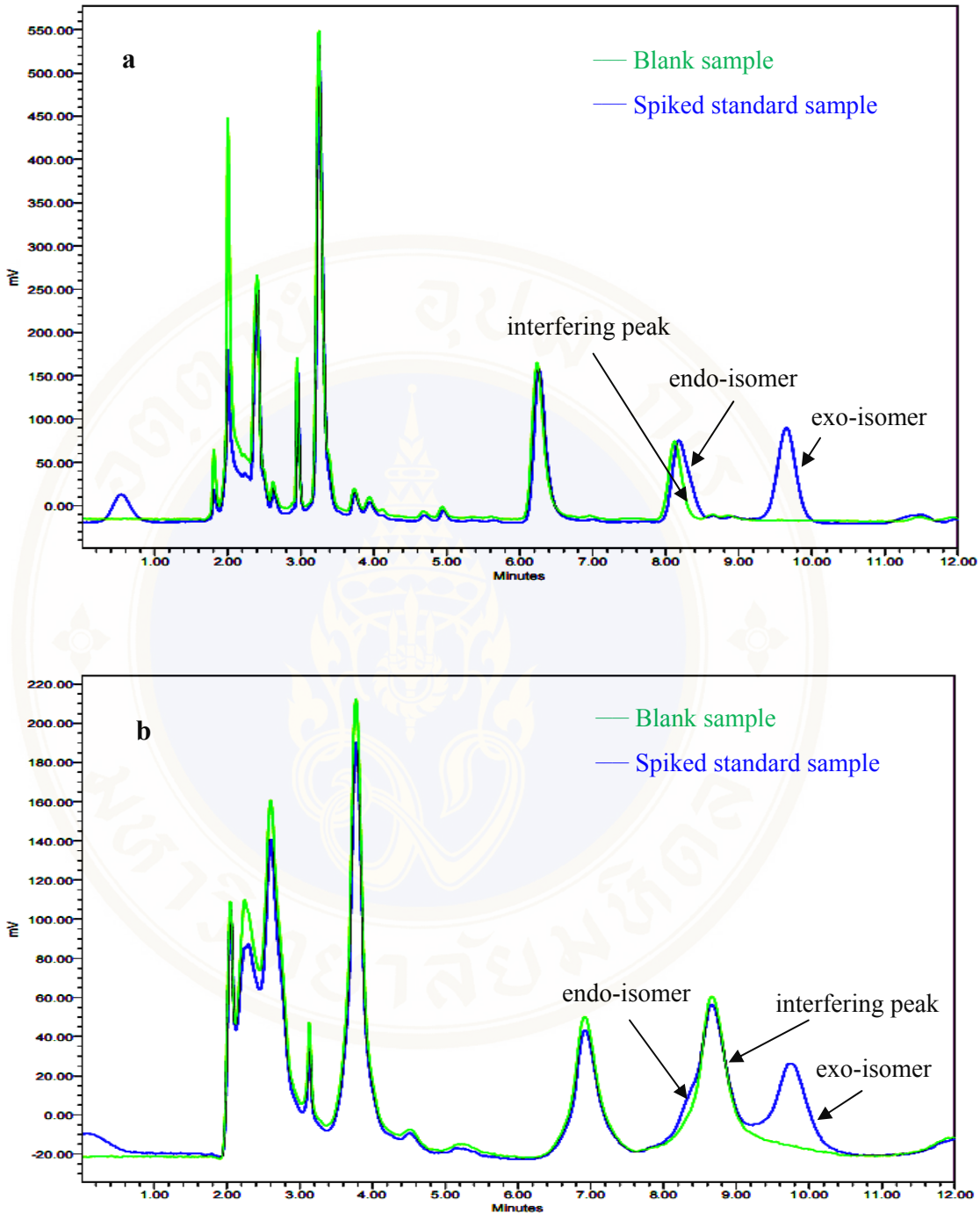

Figure 5.11 Chromatogram of liver disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 9) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively

CHAPTER VI
DISCUSSION

Accurate determination of GFR is very important for monitoring the progression of renal diseases, selecting the proper therapeutic options, and predicting the response to therapy. The gold standard for the measurement of GFR is urinary inulin clearance. Exogenously administered radioactive markers such as ^{51}Cr-ethylenediaminetetra acetic acid (^{51}Cr-EDTA), ^{99m}Tc-diethylenetriaminepenta acetic acid (^{99m}Tc-DTPA) have been used to measure GFR, but utility in routine clinical practice is hindered by risks of radioactive exposure. Serum creatinine and creatinine clearance are commonly used surrogate markers for GFR, but they are insensitive and inaccurate. Plasma clearance of iohexol has been shown to be safe and reliable method. It has shown that there a good correlation between iohexol clearance and inulin clearance, the gold standard method. Currently, the use of iohexol clearance test is widely accepted method for estimation of GFR because of the procedures to perform is easier than the gold standard (25).

HPLC-UV technique remains a method of choice of plasma iohexol measurement since this method is reliable, precise, and accurate. Moreover, this method can measure low level of iohexol thus safe dose of iohexol to be administered to patients with chronic kidney disease. Iohexol exists in two isomers, endo- and exo-isomers, and exo-isomer is the major isomer which is used for analysis. Since the methods developed by previous studies (6-8) could not completely separate these isomers and may resulted in error of data integration, therefore, improved HPLC-UV method was developed in this study for determining iohexol measurement from human plasma and influence of renal and liver diseases on this method was assessed.

6.1 Development and optimization of analytical method for iohexol determination

In order to improve the separation of iohexol by HPLC-UV method, different reverse phase columns including Alltech Alltima, Nucleosil, and Zorbax ODS were tested. The analytical method was modified from Soman et al (6) and Niculescu-Duvaz et al (7). EDTA plasma volume was reduced to 50 µl and 5% perchloric acid 800 µl for preparing 75 µl of supernatant injected into HPLC. This extraction method was utilized to extract iohexol from EDTA plasma. Based on the optimum condition of HPLC-UV system obtained by this study, iohexol was eluted into endo- and exo-isomers as reported by previous studies (6-8). Using retention time of exo-isomer, the Zorbax ODS gave the shortest retention time and Alltech Alltima gave the longest retention time. The data of retention time of 10 samples achieved by the three columns was shown in Table A1 (appendix A). No interfering peak was found at the retention time of exo-isomer for all columns. Alltech Alltima revealed the best peak shape and complete separation between endo- and exo-isomers. Overlapping of the two isomers and broader peak shape (larger peak width and peak tailing) were found in chromatograms achieved by Nucleosil and Zorbax ODS.

6.2 Validation of analytical method among the three columns

The method validation results shown that the calibration curves of the Alltech Alltima, Nucleosil and Zorbax ODS were excellently linear over iohexol concentration range of 20 - 640 µg/ml with correlation coefficients (r^2) greater than 0.9990 as shown in Figure 5.5 and Figure B1-B3 (appendix B), respectively. The data of area under the curve (AUC) for each standard iohexol concentration of the three columns was shown in Table B1-B3 (appendix B). The linearity of this study was narrow than those obtained from other works, 10 - 750 µg/ml (6) and 12.95 – 1295 µg/ml (8), respectively. Nevertheless, it was enough because iohexol concentration found in plasma after injection of Omnipaque® 5 ml (520 µg/ml) in routine practice usually range from 40 to 600 µg/ml relate to renal function of patient (8). This range was also appropriate to serve the patients who were reduced dose of iohexol

administration to avoid risk of nephrotoxicity. Other reason is the last of sampling time of iohexol after administered to patients is 300 mins, the remaining iohexol concentration at this time is very low in the blood circulation. One study found approximately 33 µg/ml at 420 min (6). Although Zorbax ODS yield the highest r^2, the least square regression of Alltech Alltima showed the best fit of linearity, with intercept value close to zero.

The inter-day and intra-day precision were expressed as % RSD and should be less than 2.0 as recommended by the ICH (30). % RSD of Alltech Alltima were excellent at all iohexol concentration (% RSD ranging from 0.94 - 1.88). Nucleosil gave the worst precision when compared to other columns, especially in the low concentration. This may cause from the column generated lowest peak height and largest peak width. Comparing with the previous studies (6-8) using Bondapak C18, Nucleosil C18 and Lichrosphere analytical columns, respectively, current method using Alltech Alltima provided a greater precision as shown in Table 6.1.

Table 6.1 The precision of previous and current methods for iohexol separation using Alltech Alltima column.

Cavalier et al [8]			Soman et al [6]		Niculescu-Duvaz et al [7]			Current study		
Conc. (µg/ml)	%RSD		Conc. (µg/ml)	%RSD	Conc. (µg/ml)	%RSD		Conc. (µg/ml)	%RSD	
	Intra-day	Inter-day		Inter-day		Intra-day	Inter-day		Intra-day	Inter-day
8.6	15.0	17.0	20.0	3.20	20.2	3.40	6.30	20.0	1.54	1.70
13.0	4.2	4.2	175.0	1.60	40.4	0.30	6.20	300.0	1.37	1.50
64.8	4.4	4.4	600.0	1.60	80.8	4.20	5.60	600.0	0.94	1.88
259.0	3.1	3.3								
518.0	2.1	2.2								
1295.0	2.4	2.7								

RSD is relative standard deviation.

Accuracy was expressed as the % recovery and all of the three columns gave the good accuracy which means of % recovery were within 100 ± 10%. Current method using Alltech Alltima provided a good accuracy at all concentrations as

previous studies (6-8), the data shown in Table 6.2. LOD and LOQ of the three columns were lower than those reported by Soman et al (6) which found to be 6 µg/ml and 10 µg/ml, respectively and reported by Cavalier et al (8) which found to be 3.08 µg/ml and 10.76 µg/ml, respectively.

Table 6.2 The % recovery of previous and current methods for iohexol separation using Alltech Alltima column.

Cavalier et al [8]		Soman et al [6]		Niculescu-Duvaz et al [7]		Current study	
Conc. (µg/ml)	% Recovery	Conc. (µg/ml)	% Recovery	Conc. (µg/ml)	% Recovery	Conc. (µg/ml)	% Recovery
8.6	96.6	20.0	92.6	50.0	100.8	20.0	100.3
13.0	101.3	175.0	98.4			300.0	99.5
64.8	100.7	600.0	100.9			600.0	99.9
259.0	102.0						
518.0	106.2						
1295.0	103.3						

Because of iohexol have isomerization, so equilibrium state was studied before using only major isomer to validate and quantitate. Similar to Soman et al (6), all the three column eluted exo-isomer as the main isomer of iohexol 80% by approximate and averages of percentages of exo-isomer peak area obtained from all columns were constant at all iohexol concentrations ranged from 20 µg/ml to 640 µg/ml. The data in Table B4 (appendix B) was indicated that no different of the percentage of exo-isomer when dissolved in mobile phase and plasma. To investigate the optimum time for reaching equilibrium state, percentages of exo-isomer in patient plasma at different times were calculated. The data in Table B5 showed that equilibrium state reached within 5 mins and did not depend on times.

6.3 Performance of the three columns

Column performances of the three columns based on the parameters; k', *As*, Rs and α were studied and showed in Table 5.5. The k' is the capacity of column to retain solute. If the column has high k' value it has high opportunity to separate interest solute from others. Many factors can influence to k' such as composition of mobile phase and column. This study used identical mobile phase so difference of column properties is the main factor to influent k' on iohexol separation. Alltech Alltima showed the highest k' that may resulted from double end-capping and polymericallic bonded phase properties while the other columns were single end-capping and monomeric chemical silane bonded phase. This technique covers the most of free silinol group and improves interaction between iohexol structure and silane (C18) inside the column. *As* is a factor that indicates the symmetry of peak. Factor influence on *As* is free silinol group in stationary phase. Broader and tailing peaks resulted from high interaction between free silinol group in stationary phase with polar part of solute. One technique which used to eliminate free silinol group for improving peak shape of polar compound is end-capping technique. Alltech Alltima eliminate free silinol group by double end-capping technique, moreover, it also use metal chelate technique so chromatogram obtained by Alltech Alltima showed a very symmetry peak shape while the other two columns were not even *As* values were within the limit. Tailing peak of exo-isomer was found in chromatogram achieved by Nucleosil because it gave the lowest *As* of exo-isomer. Rs and α values represent the separation between adjacent peak, complete separation between the two isomers leading to get accurate data validation and interpretation. The improvement of k' and use high efficiency column can improve Rs and α. Only Rs value of Alltech Alltima was accepted so the chromatogram achieved by this column showed complete separation between endo- and exo-isomer peaks. Using data obtained by standard uracil in Table C1 – C3 (appendix C), Alltech Alltima gave the same plate numbers as Nucleosil and higher than Zorbax ODS. Because higher plate numbers reflect in the higher Rs and α, Zorbax ODS gave the lowest Rs and α which the chromatogram showed large overlapping between the two isomers. Based on these parameters, Alltech Alltima showed the best performances for iohexol separation.

Retention behavior in liquid chromatography is influenced by a wide variety of physical and chemical of both the chromatographic system and solute. Differences in separation that are commonly observed among similar column have been attributed to difference in silinol activity, carbon loading, stationary phase morphology and bonding chemistry.

For isomer and other solute class with similar physical and chemical properties, molecular shape can sometime provide a basis for separation. Parameters affecting shape selectivity have been studied by previous researchers (36, 37). Shape of isomer selectivity is enhanced by increasing phase loading and longer chain length bonded phase ligands and used of polymeric phase. However, difference of shape selectivity is most strongly influenced by types of surface modification chemistry employed.

It have been recognized that C18 column provided enhanced separation of structure isomers (38, 39). An example is provided in Environmental Protection Agency (EPA) 610 method for the determination of polycyclic aromatic hydrocarbon (PAHs) from aqueous effluents. This method specifies the use of a specific C18 column. C18 columns prepared using monomeric surface modification chemistry do not exhibit the require selectivity characteristics and unable to resolve all of the components in the pollutants PAHs mixture. In general, better separation of PAHs isomers can usually be achieved by used of polymeric C18 column than with monomeric C18 column. However, the advantages offered by polymeric C18 column toward isomer separation are widely recognized involved in PAHs, as well as the separation of caroteniod isomers (40). For separation of iohexol isomers, it never used to study the separation of iohexol isomer.

Pirkle et al (41) studied on influence of end-capping on enantiomer selectivity and found that end-capping could increase selectivity of enantiomer by reducing capacity factor of first enantiomer and increasing capacity factor of second enantiomer, that enhance the separation of isomer.

6.4 Interference of renal disease and liver disease plasma

ESRD and liver disease patients can generate abnormal peak when analyzed by HPLC-UV technique (32, 33) and data of theirs influence on iohexol determination is lack. To investigate the influence of renal and liver diseases on iohexol separation, interference study has been performed on Alltech Alltima and Nucleosil C18 columns while Zorbax ODS column was excluded because its column properties quit similar to Nucleosil, larger overlapping between endo- and exo-isomers of chromatogram and previous researchers used Nucleosil in their study. Some of renal and liver patient plasma samples produced confounding peak at retention time of endo-isomer peak and also able to interfere exo- isomer peak if the resolution is poor. This phenomenon affected on accuracy of the method which determined by % recovery of the method was out the acceptable criteria. In this study, renal disease had more effects on iohexol separation than liver disease and Nucleosil was affected from these diseases greater than Alltech Alltima, especially at low iohexol concentration.

In renal disease, the interfering peak was found at the retention time of endo-isomer of the two columns. Because the resolution of chromatogram achieved by Nucleosil was poor, therefore this column was great affected as indicated by many outliers of % recoveries, especially in the low iohexol concentration (12 in 15 samples). As shown in Figure 5.6a, although interfering peak was found in chromatogram achieved by Alltech Alltima as found in the Nucleosil, the resolution of Alltech Alltima was higher, resulted in the interfering peak could not involve or overlap to exo-isomer peak. No % recovery of Alltech Alltima was out at the medium and high iohexol concentrations. Nevertheless, the chromatograms of three outlier samples achieved Alltech Alltima at the low concentration (Fig. 5.8a, Fig. D1 and Fig. D2, respectively) showed interfering peak at the retention time of exo-isomer area peak. This data indicated that though endo- and exo-isomers were completely separated, interfering peak could influence the peak area integration if it eluted at the same retention time of exo-isomer. No effect of changing in percentages between endo- and exo-isomers which found in renal patients on the accuracy of the methods because % recoveries in some samples still were out even the ratio of both isomer peaks remained constant (Table 5.7).

In liver disease, the chromatogram showed less interfering peak than the renal disease. Alltech Alltima was not affected by liver disease because interfering peak was found at only the retention time of endo-isomer peak area which completely separated from exo-isomer. This data showed that Alltech Alltima is excellent to determine iohexol in liver disease patients. Oppositely, even % recoveries at medium and high iohexol concentrations were within limit, Nucleosil was still affected by this disease at the low concentration. As found in renal disease, no effect of changing in percentages between endo- and exo-isomers on the accuracy of the methods. According to Table 5.8, bilirubin levels were not influence on iohexol separation. The possible reasons were; 1) it combines with plasma protein such as albumin and conjugates with glucoronic acid and may eliminated by protein precipitation, and 2) absorbance wavelength of bilirubin (450 or 460 nm) was not same as absorbance wavelength of iohexol (254 nm).

Interfering at endo- and exo-isomer peaks from renal and liver diseases could lead to over-estimated or under-estimated of peak area integration, depend on the interfering patterns. Over-estimated iohexol determination was found because area of interfering peak was included in exo-isomer area peak, leads to yield higher area than the true value (Figure 6.1). In contrast, under-estimated iohexol determination caused by some area of exo-isomer was included in to interfering peak and was identified to be interfering peak that resulted in losing of exo-isomer area (Figure 6.2). However, if endo- and exo-isomer peak areas were clearly separated as obtained by Alltech Alltima, blank correction for these samples could be done to decrease this problem based on the simplest way. For example, % recoveries of samples No. 3 and No. 9 achieved by Alltech Alltima in renal disease at the low iohexol concentration before corrected were 118.0% and 119.5%, respectively. After blank correction, % recoveries were 99.5% and 107%, respectively, which were in the acceptance limit.

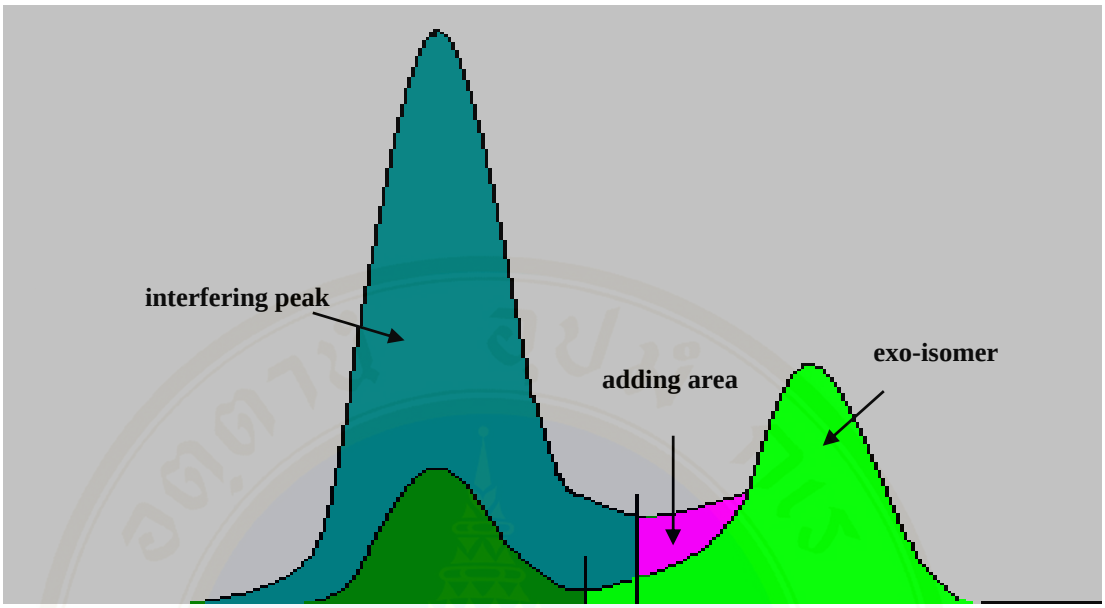

Figure 6.1 Model of over-estimated area peak integration; normal peak of iohexol which no interference was shown in green peak, interference peak was shown in blue peak and pink area is adding area of interference peak to normal peak when interference was found.

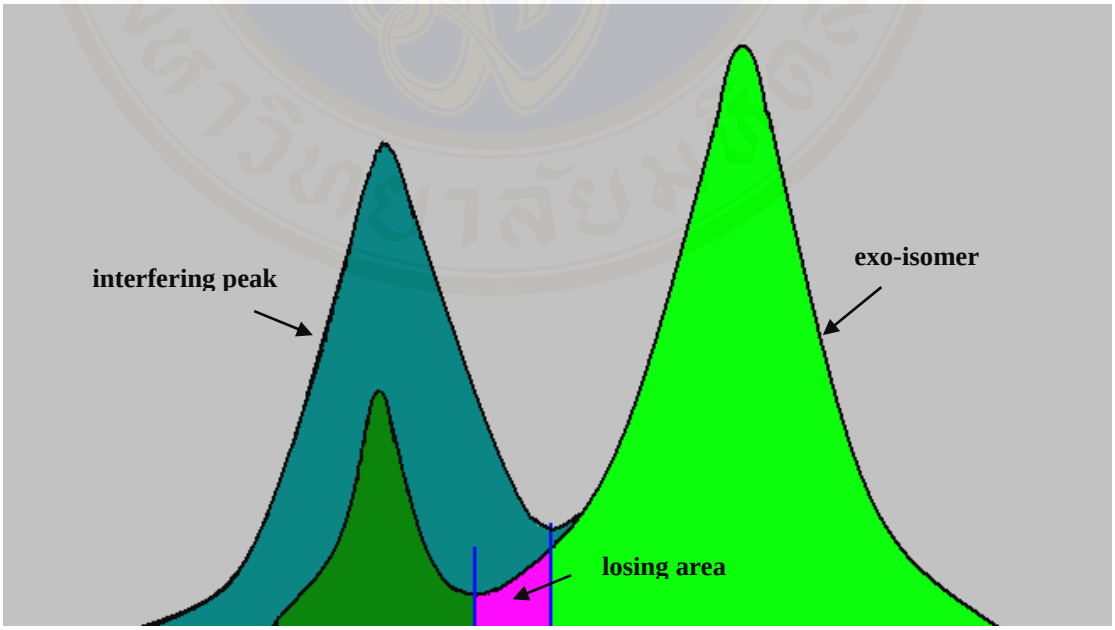

Figure 6.2 Model of under-estimated area peak integration; normal peak of iohexol which no interference was shown in green peak, interference peak was shown in blue peak and pink area is losing area of normal peak when interference was found.

CHAPTER VII
CONCLUSION

This study established an improved method for determining iohexol measurement from human plasma with HPLC-UV. The new method is simple, reliable, precise, and accurate and has the capability of being used for determination of iohexol in clinical settings. Based on the results, Alltech Alltima C18 column; the column which eliminated silinol group by double end-cap and bonded silane phase by polymericallic technique was identified for excellent discrimination between endo- and exo-iohexol. Moreover, it showed better peak shape that is easy to integrate leading to better in accuracy and precision than other columns.

Some sample from renal and liver patients could generate interfering peak at the retention time of endo-isomer of iohexol and overlaid to exo-isomer. Only Alltech Alltima column was capable to clearly distinguishing interfering peaks from exo-isomer leading to good accuracy and precision in renal and liver diseases. Therefore, Alltech Alltima C18 column provide a practical alternative to other commonly used C18 columns for iohexol quantification. However, interfering peak of renal and liver diseases, may cause from drugs which the patients use to therapy or toxin substance cumulated in the body, influences on chromatogram in some sample. This study still doesn’t know the origin of interfering so further studies have to perform for investigation interfering peaks and clinical application of this method on glomerular filtration rate determination in nephrological practice.

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APPENDICES

APPENDIX A

Table A1 Retention time of 10 samples achieved by the three columns.

No.	Retention time (mins)		
	Alltech Alltima	**Nucleosil C18**	**Zorbax ODS**
1	9.43	9.54	5.45
2	9.43	9.52	5.60
3	9.45	9.57	5.60
4	9.45	9.53	5.61
5	9.46	9.59	5.61
6	9.48	9.62	5.62
7	9.49	9.61	5.62
8	9.50	9.56	5.63
9	9.43	9.61	5.63
10	9.48	9.50	5.63
$\bar{x}$	9.46	9.56	5.60
SD	0.03	0.04	0.05
%CV	0.30	0.44	0.94

APPENDIX B

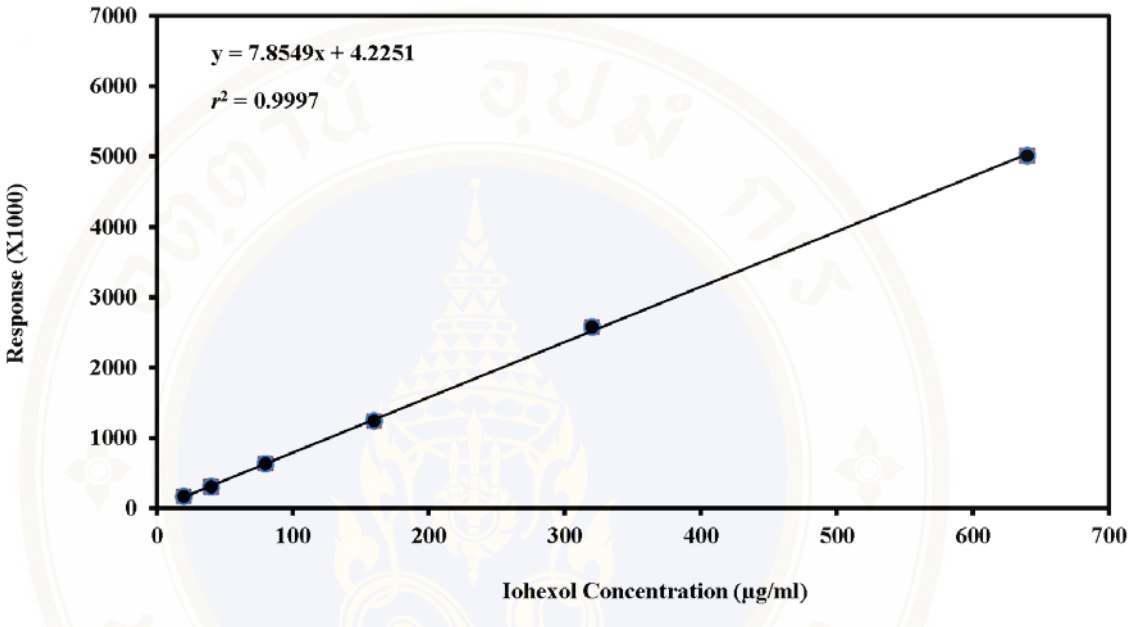

Figure B1 Calibration curve of Alltech Alltima.

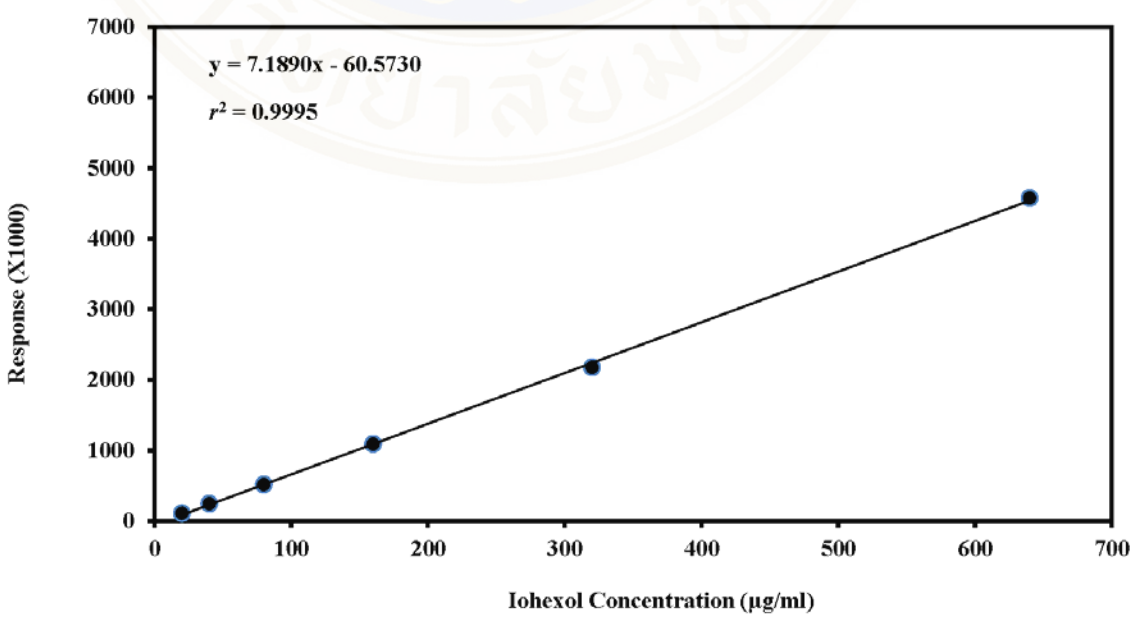

Figure B2 Calibration curve of Nucleosil C18.

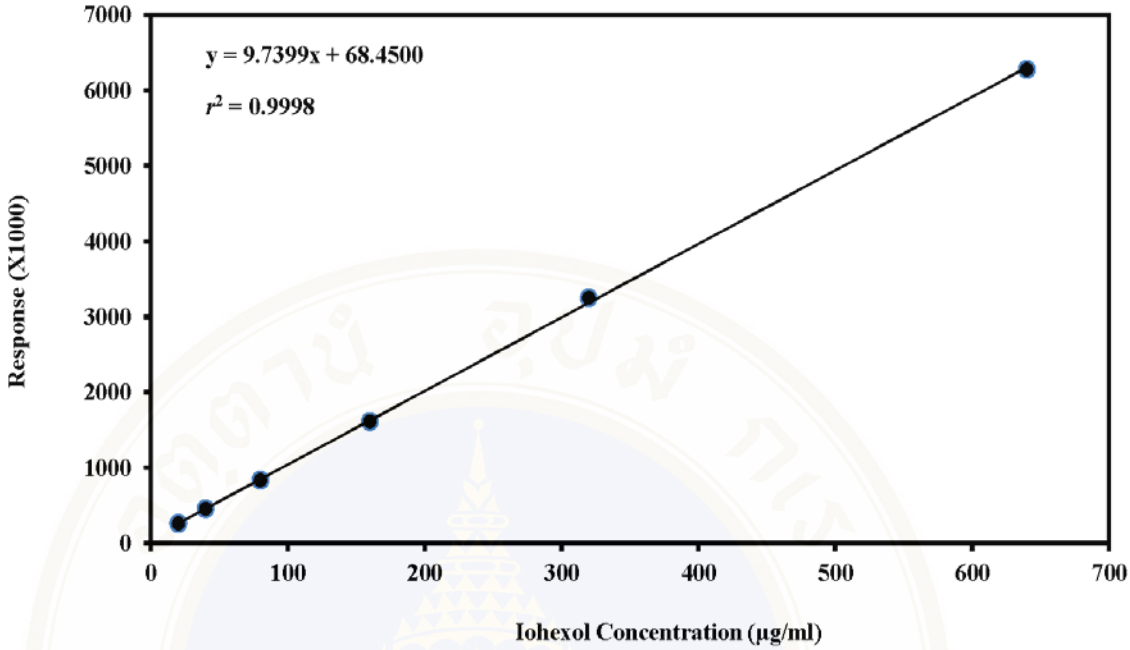

Figure B3 Calibration curve of Zorbax ODS.

Table B1 Area under the curve (AUC) of six concentrations of standard to prepare calibration curve achieve by Alltech Alltima.

Conc. (µg/ml)	AUC of iohexol standards			$\bar{x}$ ± SD
	Run 1	Run 2	Run 3	
20	1671592	1637353	1698837	1669261 ± 30808
40	3128147	3085584	3081154	3028295 ± 25947
80	6278438	6060227	6556288	6298318 ± 248627
160	12748517	12058239	12347397	12384718 ± 346649
320	25082419	26074337	25689947	25748901 ± 697829
640	49795575	50087713	50405754	50096347 ± 305181

Table B2 Area under the curve of six concentration of standard to prepare calibration curve achieve by Nucleosil C18.

Conc. (µg/ml)	AUC of iohexol standards			$\bar{x}$ ± SD
	Run 1	Run 2	Run 3	
20	1073932	1101501	1029730	1068388 ± 36205
40	2457827	2499369	2287314	2414837 ± 112374
80	5118079	5412138	4868774	5132997 ± 271989
160	10864720	11032501	10749505	10882242 ± 142309
320	22100299	21659103	21380165	21713189 ± 363100
640	45605140	47189939	44412504	45735861 ± 1393324

Table B3 Area under the curve of six concentration of standard to prepare calibration curve achieve by Zorbax ODS.

Conc. (µg/ml)	AUC of iohexol standards			$\bar{x}$ ± SD
	Run 1	Run 2	Run 3	
20	2793432	2489303	2502471	2595069 ± 171914
40	4803848	4753450	4065784	4541027 ± 412343
80	8284833	8205137	8480428	8323466 ± 141653
160	16508831	15760697	16045521	16105016 ± 377599
320	33698972	31494789	32351142	32514967 ± 1111186
640	63293706	64250990	62494907	63346534 ± 879233

Table B4 Percent of exo-isomer dissolved in mobile phase and in plasma*.

No	In mobile phase	In plasma
1	80.1	80.3
2	81.3	81.5
3	81.7	81.3
4	81.6	81.0
5	81.6	80.1
6	81.5	81.7
7	81.3	81.5
8	81.3	81.2
9	82.0	82.0
10	82.0	81.3
$\bar{x}$	**81.4**	**81.2**
SD	**0.5**	**0.7**

*Analyzed by Alltech Alltima

Table B5 Percent of exo-isomer in patient plasma at different times.

Min / No	Percentage of exo-isomer										
	5	**10**	**20**	**30**	**45**	**60**	**105**	**120**	**180**	**240**	**300**
1	82.2	82.5	82.8	83.0	83.2	82.8	82.8	82.8	82.8	82.5	82.0
2	81.4	81.8	81.5	81.2	81.2	80.9	80.3	80.1	79.6	79.3	79.0
3	80.6	80.0	80.0	80.0	80.2	80.9	81.5	81.7	82.1	82.7	83.0
4	80.3	80.2	80.0	80.6	79.5	79.6	81.3	81.5	82.0	82.0	82.3
5	80.8	80.7	80.9	80.9	80.9	81.0	81.0	81.2	81.3	81.3	81.5
$\bar{x}$	**81.1**	**81.1**	**81.1**	**81.1**	**81.0**	**81.1**	**81.5**	**81.5**	**81.6**	**81.6**	**81.6**
SD	**0.9**	**1.2**	**1.4**	**1.1**	**1.6**	**1.3**	**1.0**	**1.1**	**1.4**	**1.6**	**1.8**

*Analyzed by Alltech Alltima.

APPENDIX C

Table C1 Retention time and efficiency of mixing compound (first peak is uracil, second peak is phenol, third peak is N,N-diethyl-m-toluamide and the last peak is toluene) in 58/42 acetonitrile/ water, v/v, flow rate 1.0 ml/min, UV detector set at 254 nm and achieved by Alltech Alltima.

Substance	Retention time (minute)	Efficiency (plates/meter)
Uracil	2.45	82000
Phenol	4.00	118246
N,N- diethyl-m-toluamide	6.50	120675
Toluene	11.72	113118

Table C2 Retention time and efficiency of mixing compound (first peak is uracil, second peak is phenol, third peak is N,N-diethyl-m-toluamide and the last peak is toluene) in 58/42 acetonitrile/ water, v/v, flow rate 1.0 ml/min, UV detector set at 254 nm and achieved by Nucleosil C18.

Substance	Retention time (minute)	Efficiency (plates/meter)
Uracil	2.83	81961
Phenol	4.82	113061
N,N- diethyl-m-toluamide	7.87	114046
Toluene	12.95	103835

Table C3 Depict retention time and efficiency of mixing compound (first peak is uracil, second peak is phenol, third peak is N,N-diethyl-m-toluamide and the last peak is toluene) in 65/35 acetonitrile/ water, v/v, flow rate 1.0 ml/min, UV detector set at 254 nm and achieved by Zorbax ODS.

Substance	Retention time (minute)	Efficiency (plates/meter)
Uracil	3.02	70666
Phenol	3.50	68258
N,N- diethyl-m-toluamide	5.15	70352
Toluene	5.88	70550

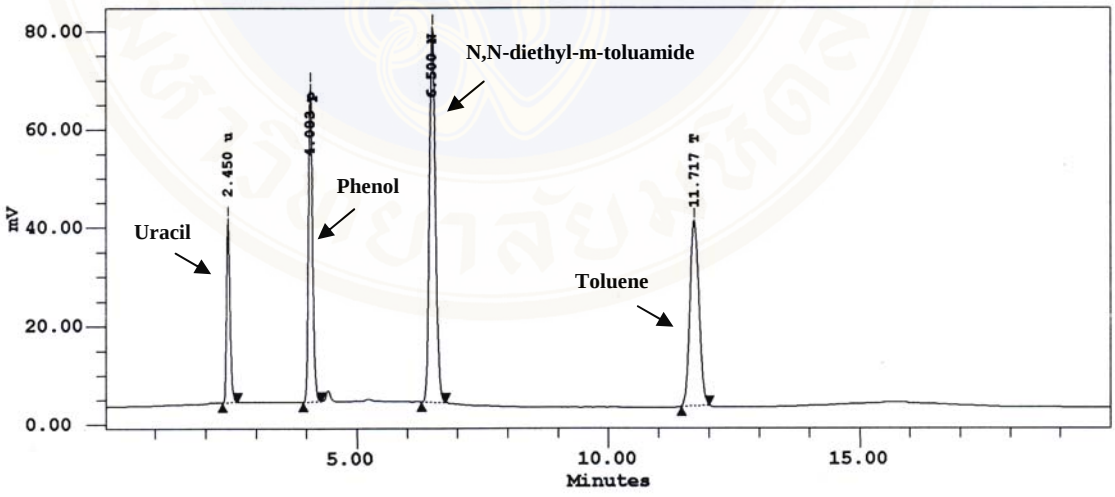

Figure C1 Chromatogram of mixing compound (first peak is uracil, second peak is phenol, third peak is N,N-diethyl-m-toluamide and the last peak is toluene) in 58/42 acetonitrile/water, v/v, flow rate 1.0 ml/min, UV detector set at 254 nm and achieved by Alltech Alltima.

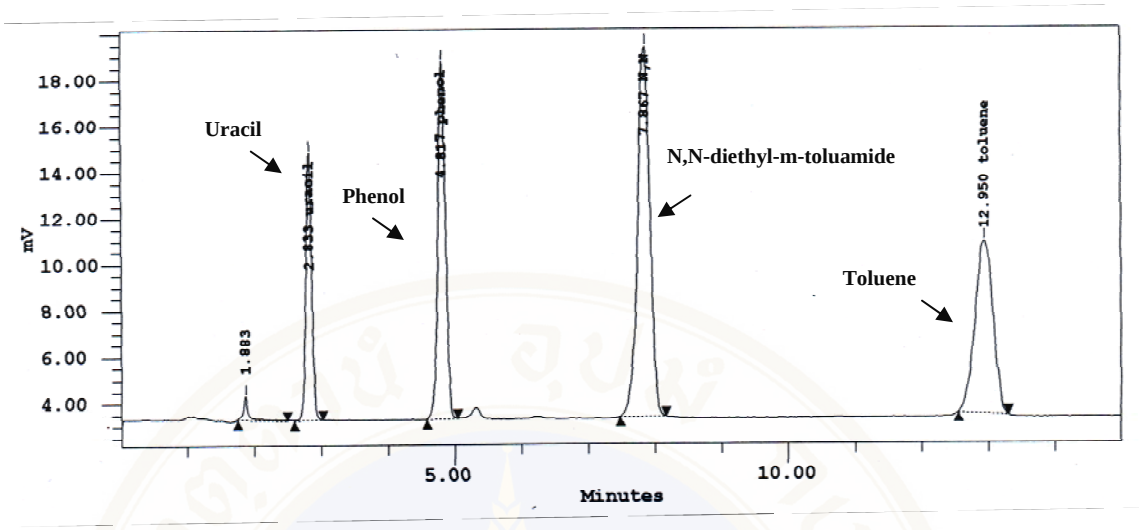

Figure C2 Chromatogram of mixing compound (first peak is uracil, second peak is phenol, third peak is N,N-diethyl-m-toluamide and the last peak is toluene) in 58/42 acetonitrile/ water, v/v, flow rate 1.0 ml/min, UV detector set at 254 nm and achieved by Nucleosil C18.

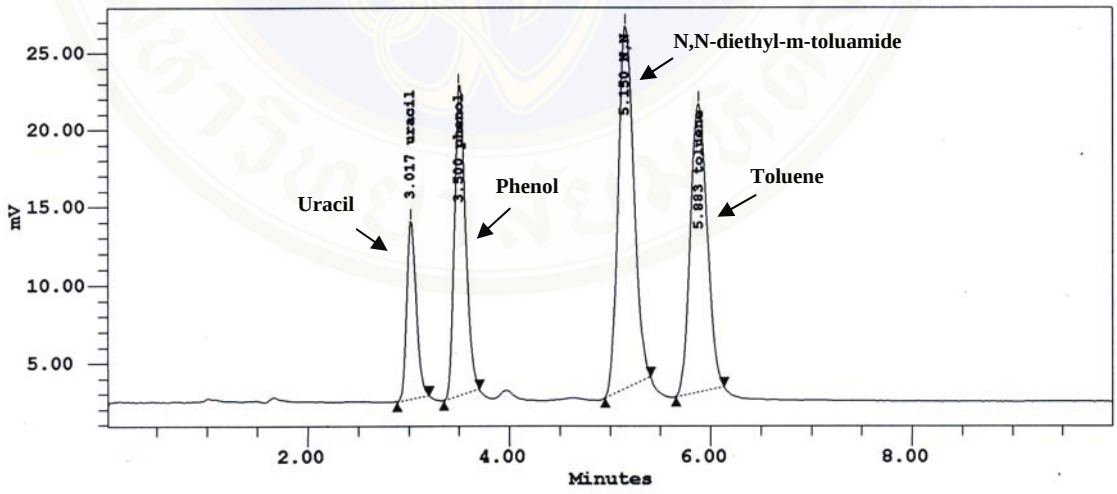

Figure C3 Chromatogram of mixing compound (first peak is uracil, second peak is phenol, third peak is N,N-diethyl-m-toluamide and the last peak is toluene) in 65/35 acetonitrile/ water, v/v, flow rate 1.0 ml/min, UV detector set at 254 nm and achieved by Zorbax ODS.

APPENDIX D

Table D1 Observed iohexol concentrations which used to calculate % recovery for exo-isomer in renal patient plasma.

No.	Observed iohexol concentrations (µg/ml)					
	Alltech Alltima			Nucleosil C18		
	Low	Medium	High	Low	Medium	High
1	20.6	305.0	582.5	23.2	302.9	603.3
2	20.5	298.6	592.0	16.9	283.8	621.1
3	23.6	300.3	619.9	25.1	277.1	599.8
4	21.2	291.0	591.7	16.6	295.8	587.6
5	19.4	293.9	601.6	16.2	299.7	642.5
6	19.0	293.7	604.3	21.8	311.7	629.5
7	19.5	286.4	606.6	17.9	255.9	600.2
8	20.1	300.5	597.8	16.4	317.4	585.5
9	23.9	293.1	615.1	36.7	286.8	586.4
10	20.0	304.6	626.3	28.3	267.6	548.9
11	16.8	319.4	626.8	18.2	287.5	601.2
12	20.0	298.7	624.2	19.9	283.6	600.6
13	20.2	303.7	619.4	17.0	290.9	621.8
14	19.7	311.5	608.2	26.2	314.1	588.7
15	20.1	308.8	627.2	22.9	318.4	612.1
$\bar{x}$	20.4	300.0	601.3	21.1	289.3	606.8
SD	1.8	8.60	11.8	6.0	16.9	19.3
% RSD	9.00	2.9	2.0	28.6	5.8	3.19
% Recovery ± SD	100.5 ± 8.5	100.2 ± 2.9	101.6 ± 2.4	107.8 ± 28.8	97.6 ± 6.1	100.3 ± 3.8

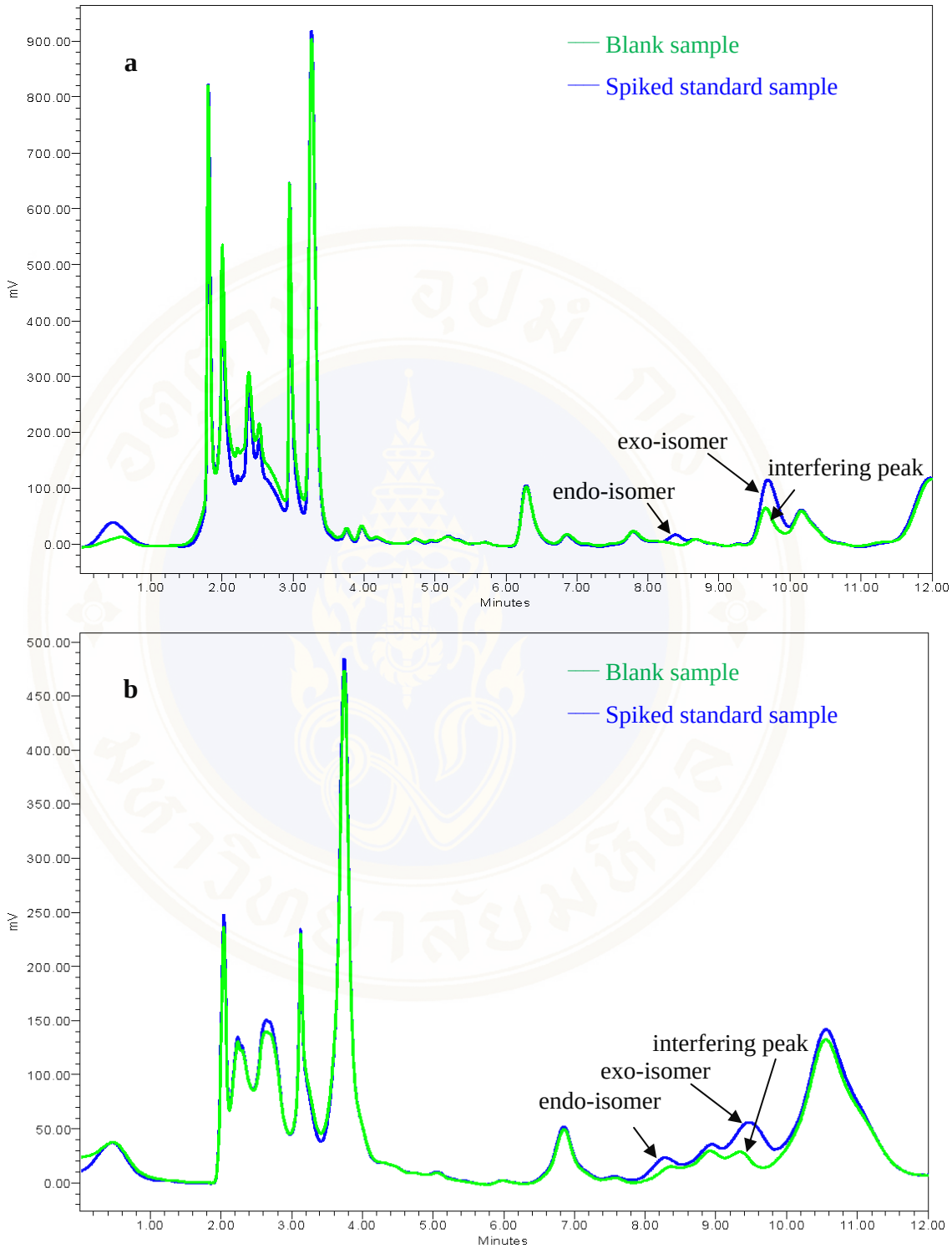

Figure D1 Chromatogram of renal disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 9) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

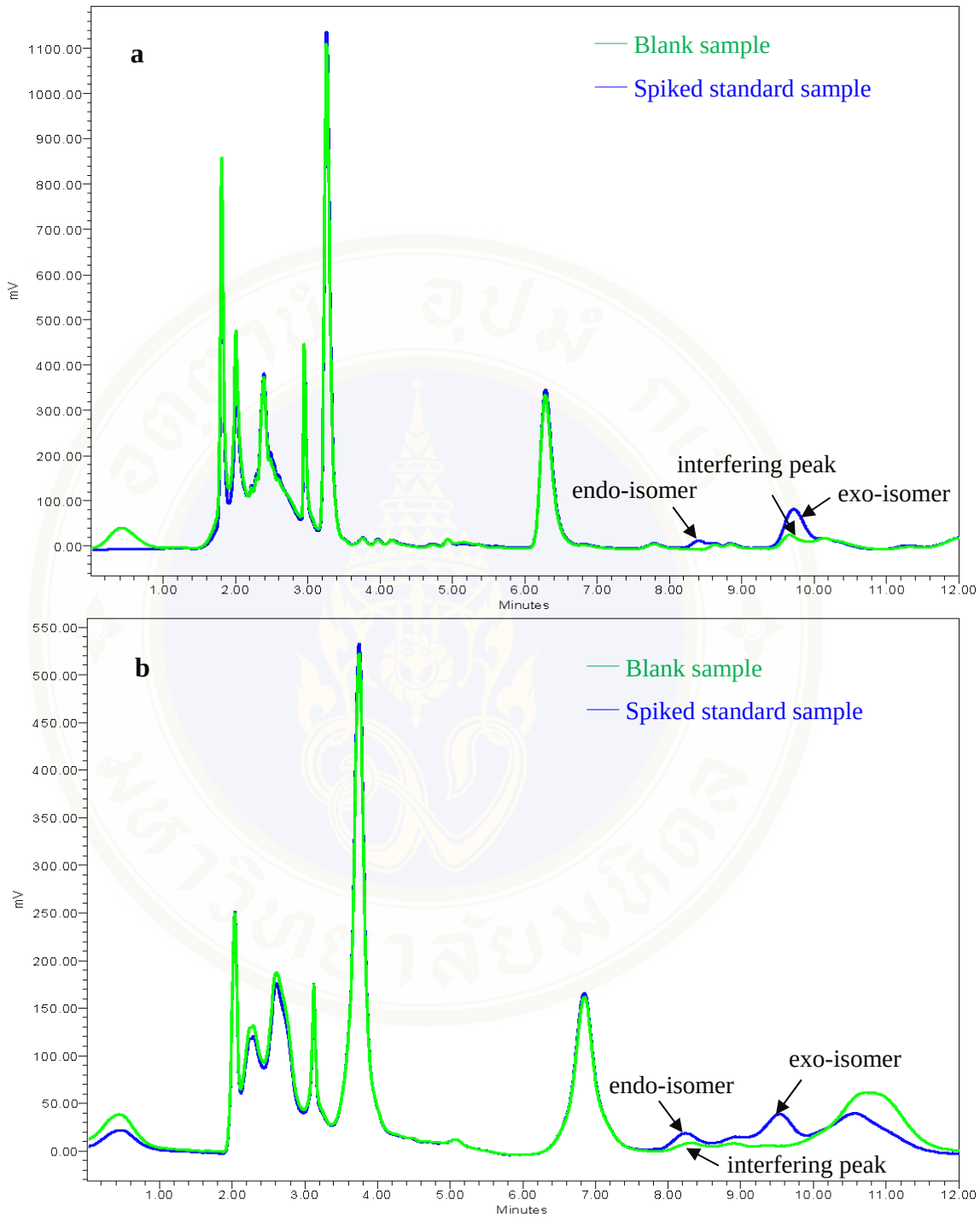

Figure D2 Chromatogram of renal disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 11) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

Table D2 Observed iohexol concentrations which used to calculate % recovery for exo-isomer in liver patient plasma.

No.	Total bilirubin (mg/dl)	% Recovery					
		Alltech Alltima			Nucleosil C18		
		Low	Medium	High	Low	Medium	High
1	0.5	20.9	320.5	617.7	20.7	320.1	617.9
2	0.6	19.4	325.7	584.8	18.4	324.6	583.2
3	0.8	19.5	315.6	621.2	15.2	312.2	622.5
4	0.9	20.2	300.0	596.1	19.5	297.6	616.3
5	1.0	19.2	300.9	586.7	18.5	301.3	578.6
6	1.0	18.2	307.3	582.7	22.8	302.4	600.7
7	1.2	19.8	309.3	600.9	22.5	306.7	622.5
8	1.4	20.6	306.6	583.7	19.2	305.8	581.8
9	2.5	21.0	307.1	591.7	24.7	297.8	605.2
10	3.6	21.7	301.2	593.0	20.1	319.7	633.0
11	9.7	18.1	300.6	600.5	20.9	312.3	619.2
12	10.7	19.3	312.0	599.5	24.3	317.8	633.6
13	20.5	20.5	292.3	592.5	21.6	305.3	634.7
14	23.7	19.7	307.1	601.2	19.8	301.2	611.9
15	27.3	19.2	323.1	579.6	16.2	325.2	574.5
$\bar{X}$		19.8	308.7	595.5	20.3	310.0	60
SD		1.0	9.3	12.1	2.7	9.5	22.5
% RSD		5.9	3.0	2.0	12.0	3.0	3.7
% Recovery ± SD		99.1±5.0	102.9±3.1	99.2±2.0	101.5±13.4	103.3±3.2	101.5±3.5

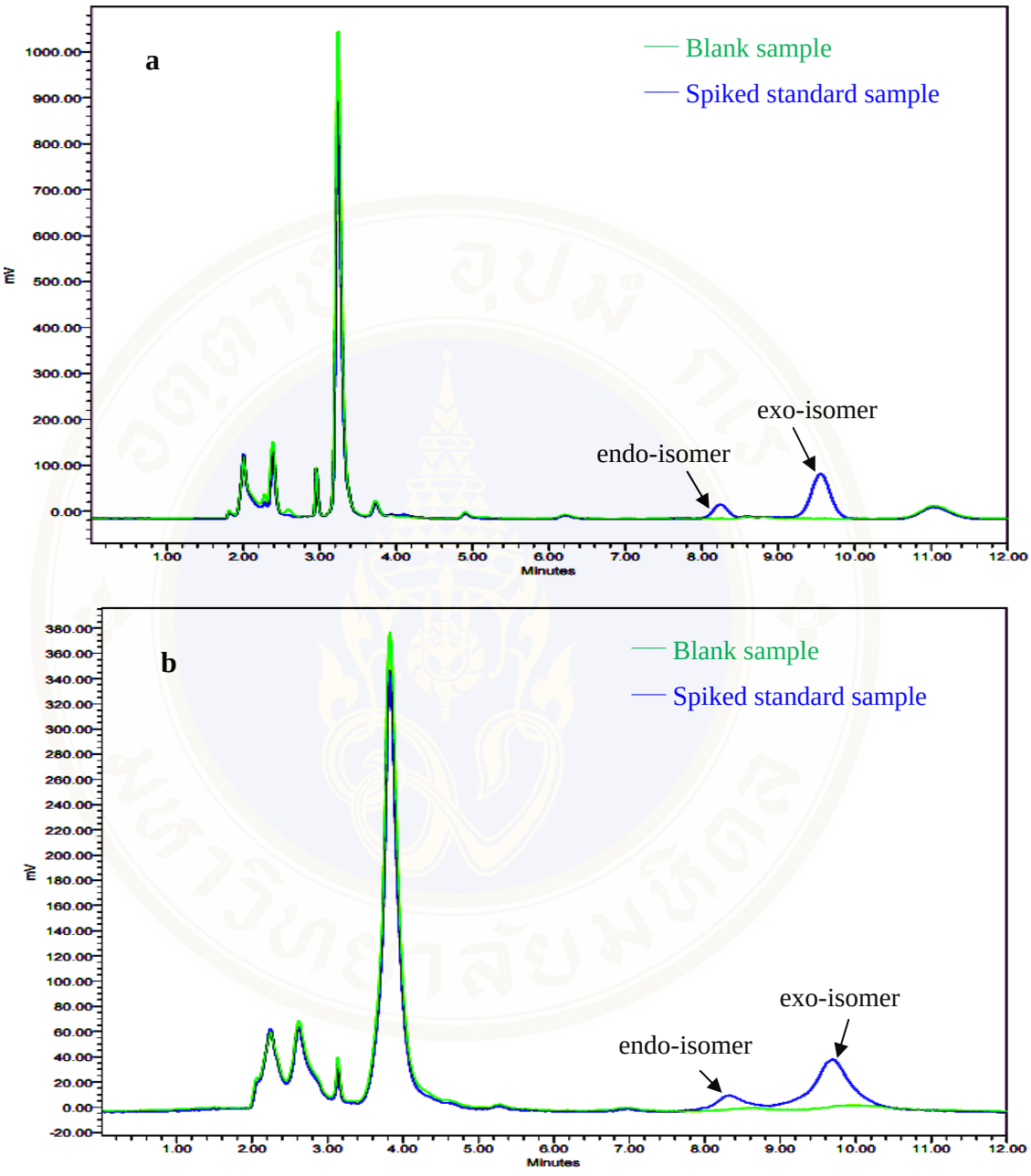

Figure D3 Chromatogram of liver disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 3) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

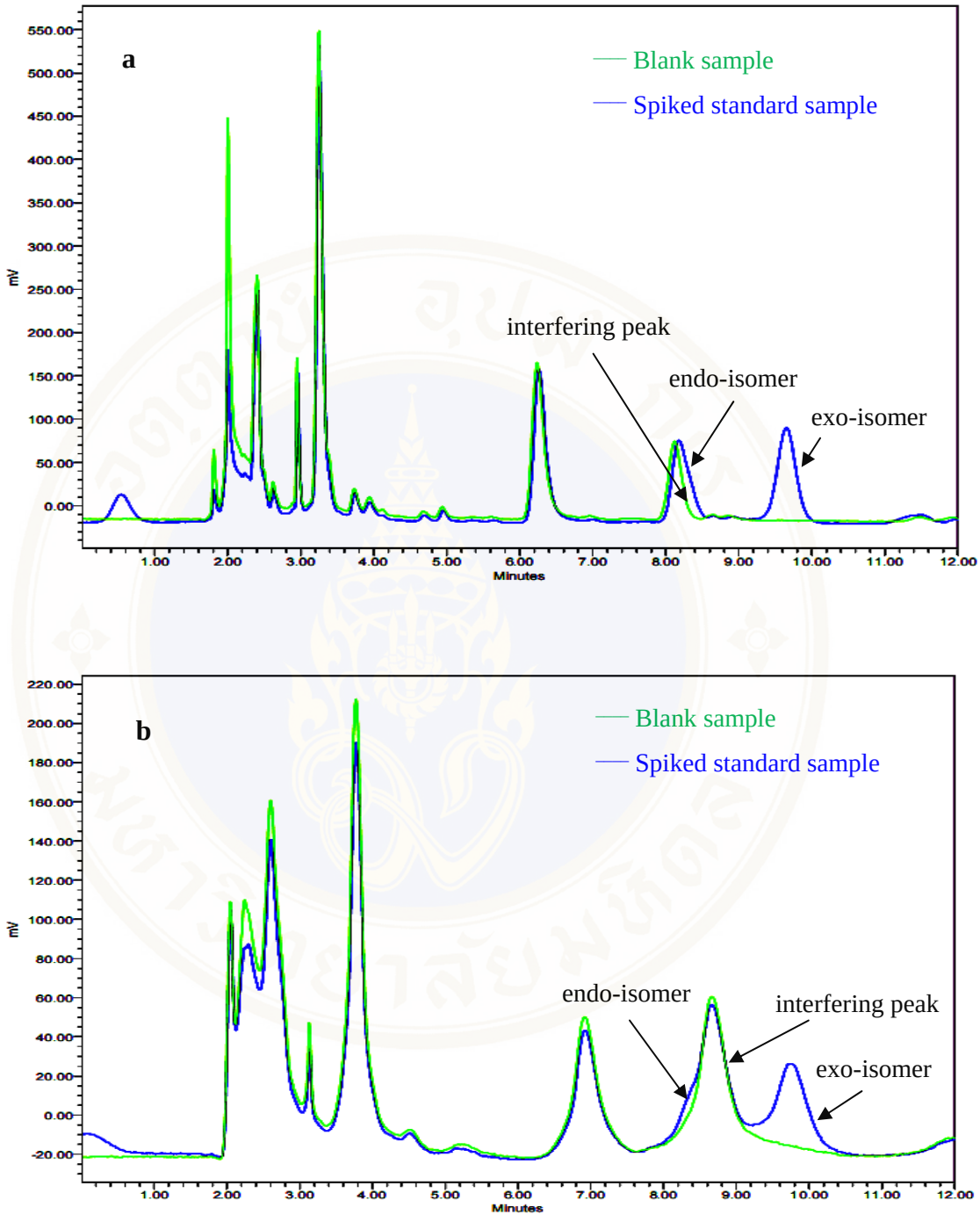

Figure D4 Chromatogram of liver disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 9) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

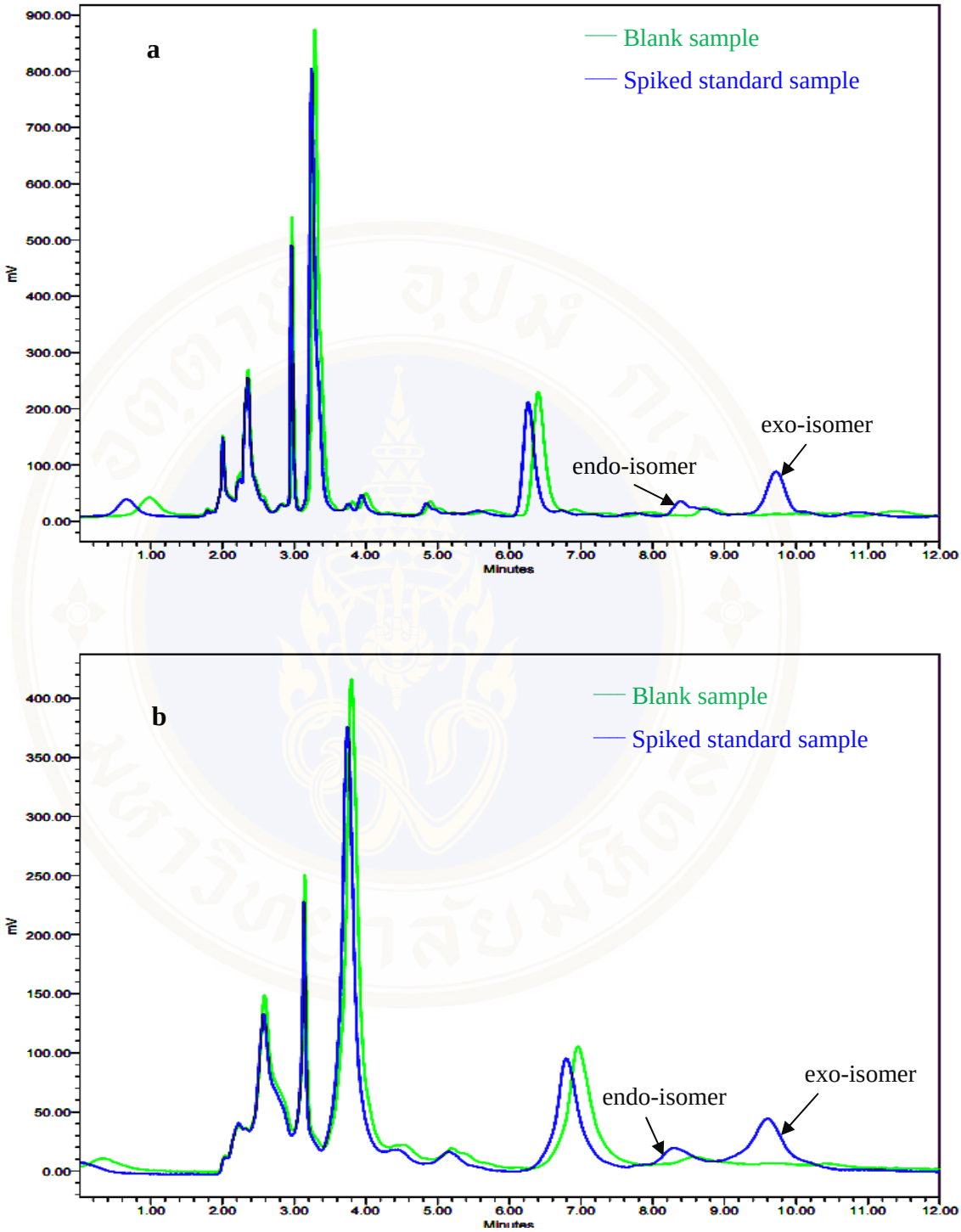

Figure D5 Chromatogram of liver disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 12) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

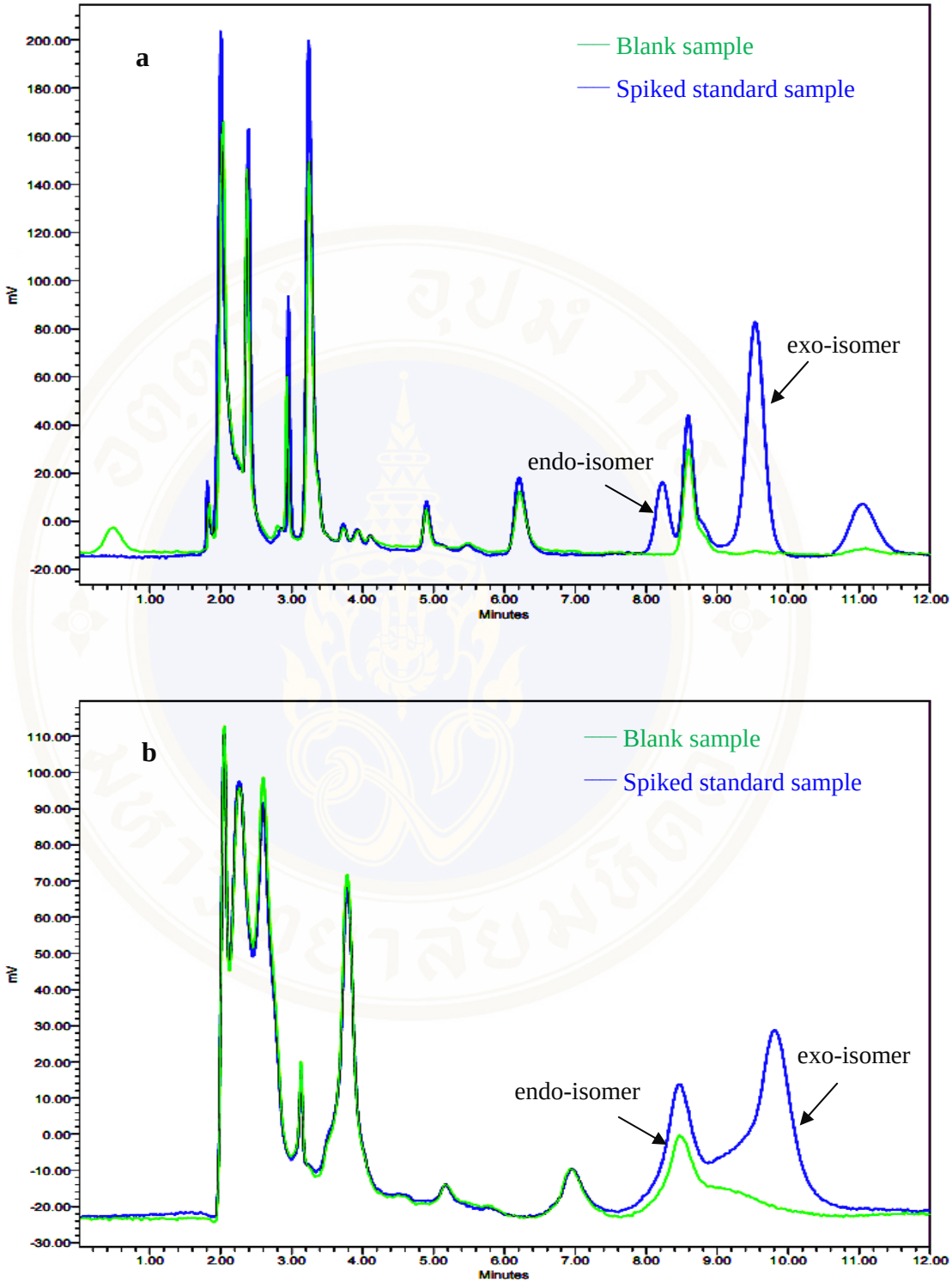

Figure D6 Chromatogram of liver disease plasma with spiked 20 µg/ml iohexol concentration (sample no. 15) achieved by Alltech Alltima (a) and Nucleosil C18 (b), respectively.

BIOGRAPHY

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