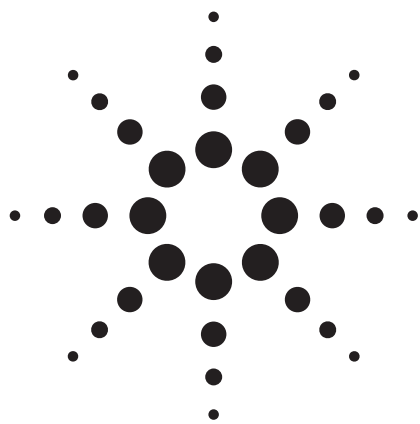




使用 SPE 混合型强阳离子交换聚合物树脂（安捷伦 SampliQ SCX）对血浆中的酸性、碱性和中性药物进行分离。

Application Note

Drug Discovery

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Abstract

本研究开发了一种从加标的人体血浆样品中同时提取多种药物（安非他明、对乙酰氨基酚、对甲苯酰胺、间甲苯胺和苯巴比妥）的方法。此过程采用混合型强阳离子交换树脂安捷伦 SampliQ SCX 进行了固相萃取。固相萃取物的色谱分离和分析条件如下：流动相为 30% 甲醇和 70% 磷酸二氢钾（以等度条件运行）；色谱柱为安捷伦 ZORBAX Eclipse Plus C18 (4.6 mm×75 mm, 3.5 μm)；流速为 1 mL/min；二极管阵列检测器设置在 210 nm。所有分析物的分析结果回收率都较高 (> 80%) 且具有高度可重现性。对乙酰氨基酚的检测限 (LOD) 和定量限 (LOQ) 分别为 0.39 和 0.71 μg/mL，安非他明为 0.84 和 1.87 μg/mL，间甲苯胺为 0.36 和 1.06 μg/mL，对甲苯酰胺为 0.66 和 0.70 μg/mL，苯巴比妥为 0.80 和 1.89 μg/mL。



Agilent Technologies

Introduction

Sample preparation prior to chromatographic analysis presents a major challenge for the determination of drugs and their metabolites in complex matrices such as biological fluids (for example, blood, plasma, urine, serum, saliva). Drugs normally exhibit a diverse polarity with acidic, basic or neutral functionalities depending on the pH of the matrix. Liquid-liquid extraction (LLE) and solid phase extraction (SPE) have traditionally been employed for the extraction of drugs, their metabolites and endogenous compounds from plasma [1-2]. Their quantification at a low concentration has proven to be a difficulty in pharmaceutical analysis. SPE is however the preferred method, as it is not only employed for class fractionation but also for trace enrichment and purification. Available commercial sorbents such as chemically modified silica gel, polymer and graphitized or porous carbon are used [3]. These offer separations based on normal phase, reversed phase, ion exchange and mixed mode ion exchange (combination of reversed phase and ion exchange) sorbents. The mixed mode sorbents have proven to give cleaner extracts and better separations than standard reversed phase or ion exchange sorbents as they take advantage of both the ion exchange and hydrophobic interactions [4-5]. For the extraction of compounds with a wide variety of polarity, polymeric sorbents have proven to be superior to other sorbents (for example, alkylated silica) and are therefore the choice of sorbent for this study [6-8].

In this application note a method employing solid phase extraction is developed for the fractionation of acidic, basic and neutral drugs from plasma with Agilent SampliQ SCX, a mixed mode strong cation exchange polymer. The resin is a sulfonic acid modified divinyl benzene polymer that exhibits both cation exchange and reversed phase behavior. In addition, it provides excellent reproducibility and enables a simple extraction protocol. Specific drugs (Figure 1) have been used as representatives of the three classes of drugs for example p-toluamide (acidic), amphetamine (basic) and acetaminophen (neutral).

Experimental

Chemicals

Acetaminophen, phenobarbital, p-toluamide, amphetamine, m-toluidine and ranitidine (IS) were purchased from Sigma-Aldrich Chemicals (St. Louis, MO, USA). Phosphoric acid, formic acid and potassium hydroxide were purchased from Merck Chemicals (Gauteng, South Africa) while the HPLC grade methanol was from Merck KGaA (Darmstadt, Germany), dipotassium hydrogen phosphate and potassium dihydrogen

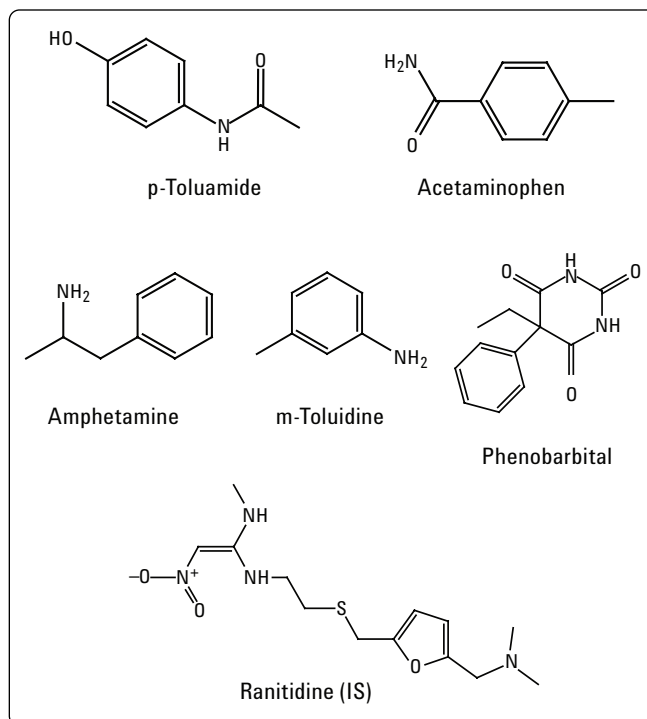


Figure 1. Structures of drugs used in present study.

phosphate were purchased from Saarchem Analytical (Krugersdorp, South Africa).

The mobile phase was prepared with ultrapure water (18.2 M Ωcm) from a MilliQ system by Millipore (Milford, Mass, USA) and filtered through a Whatman membrane filter (47 mm diameter and 0.2 μm pore size). The stock solutions of the drugs and the internal standard were prepared in methanol (1000 μg/mL) and kept at 4 °C while the working solutions were prepared daily by diluting the stock solutions, to appropriate concentrations, also in methanol. The plasma (ECZ HQ Donation 5497780, O⁺) was from SANBS (Port Elizabeth, South Africa).

Instrumental

The analysis was performed on an Agilent 1200 Series HPLC composed of a binary pump and a diode array detector (DAD) set at λ = 210 nm. Separation of the compounds was achieved on an Agilent ZORBAX Eclipse Plus C18 column (4.6 mm x 75 mm, 3.5 μm, p/n 959933-902, Agilent Technologies, Santa Clara, CA). The data was processed by HPLC 2D ChemStation software. The solid phase extraction cartridges were Agilent SampliQ SCX, 1-mL/30 mg, p/n 5982-3213, a polymeric strong cation exchanger with 25–35 μm average particle sizes. A Jenway 3510 pH meter (London, UK) was employed for pH adjustments.

Sample pretreatment: SPE procedure

The plasma sample (1 mL) was hydrolyzed with 1% formic acid (3 mL) for 30 min. The sample, spiked with drugs, was then loaded onto the SampliQ SCX cartridges, as described in Figure 2. An internal standard, 50 μ L of the ranitidine stock solution, was added to each SPE fraction. A blank plasma sample (1 mL) was carried through the procedure also.

Separation and analysis

The reversed-phase chromatographic conditions were as shown in Table 1. All drugs were separated within five minutes.

Table 1. HPLC Conditions

Column:	Agilent ZORBAX Eclipse Plus C18 4.6 mm $\times$ 75 mm, 3.5 μ m
Flow rate:	1.5 mL/min
Column temperature:	35 $^{\circ}$ C
Injection volume:	5 μ L
Mobile phase:	Isocratic elution: 30/70 A/B A: CH ₃ OH B: 25 mM K ₂ HPO ₄ , pH 7

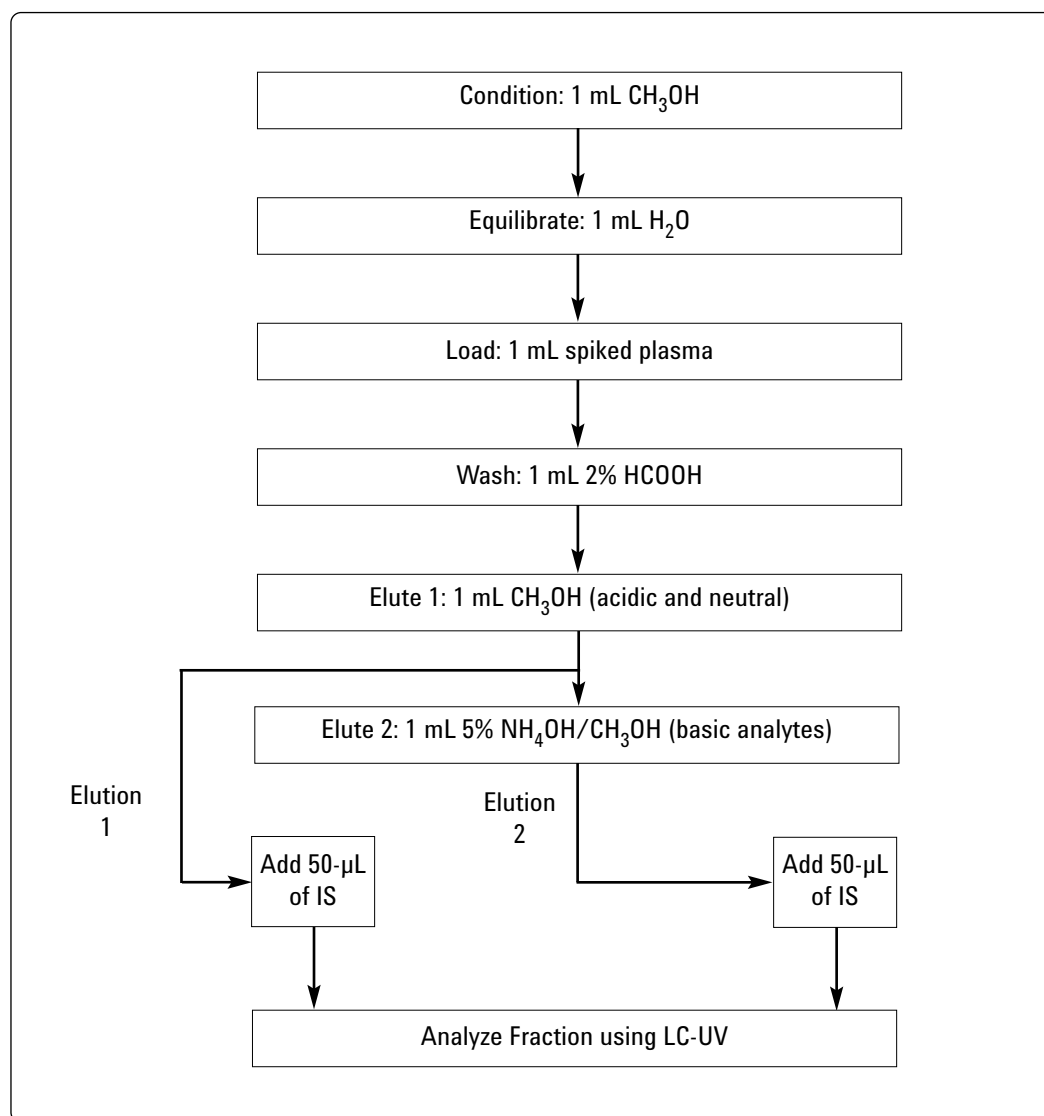


Figure 2. SPE procedure.

Results and Discussion

Separation

The standard mixture of the analytes was separated with the set chromatographic conditions (Table 1) and the chromatogram is reported in Figure 3.

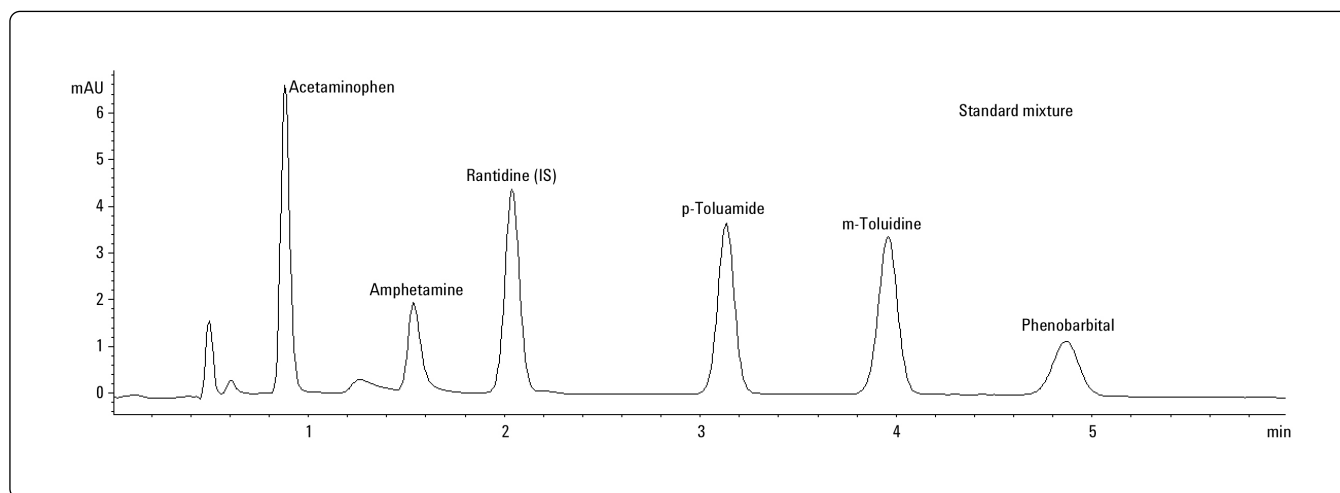


Figure 3. Chromatogram of a standard mixture (7 $\mu\text{g/mL}$): [1] acetaminophen [2] amphetamine [3] p-toluamide [4] m-toluidine [5] phenobarbital rantidine (IS).

Analysis of standard solutions

Calibration curves were processed at 0–10 $\mu\text{g/mL}$ concentration ranges for all the analytes. They were linear with coefficient of regression (r^2) greater than 0.999 as shown in Figure 4.

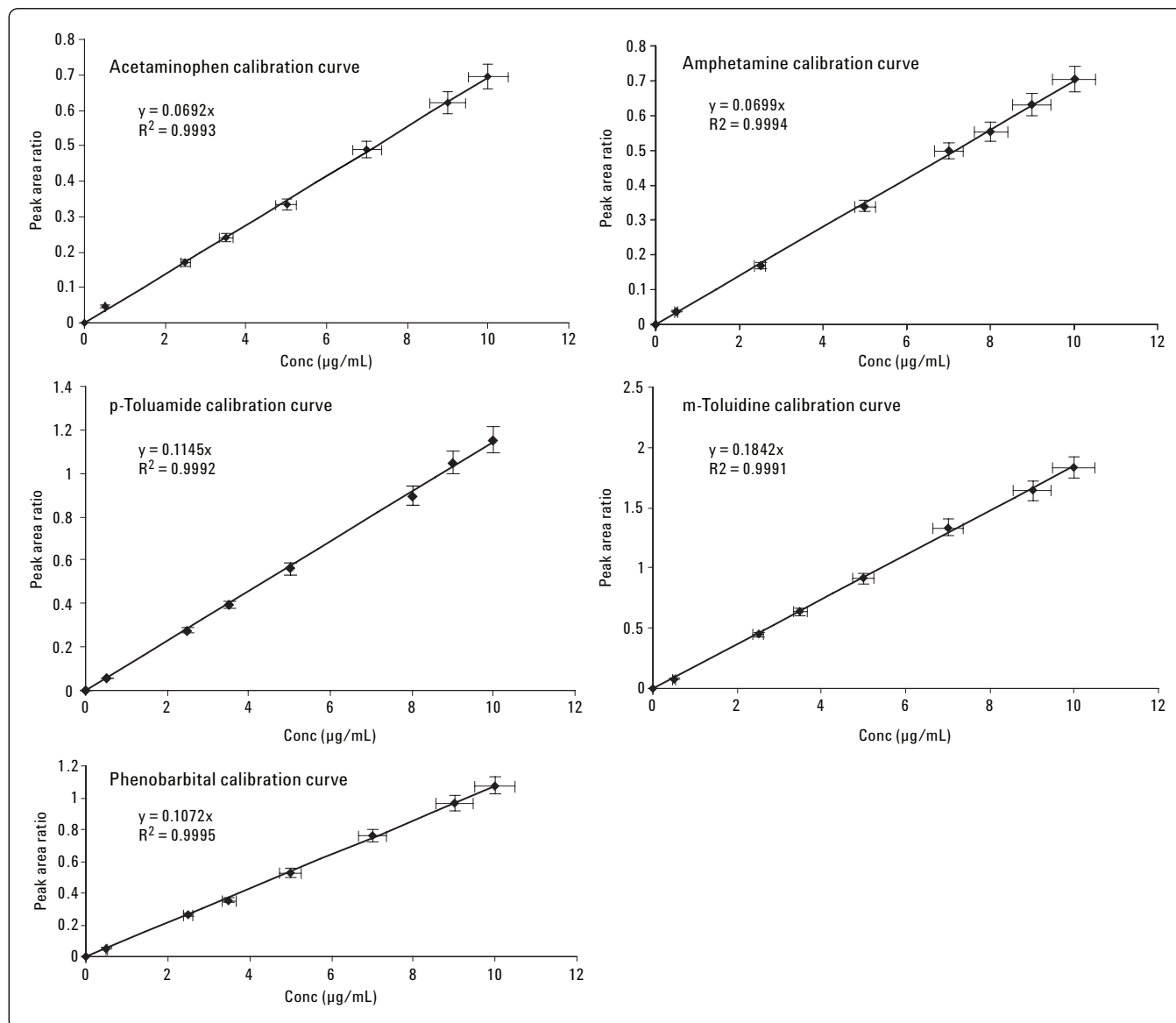


Figure 4. Calibration curves of the standards.

Solid phase extraction procedure

A mixed mode strong cation exchange sorbent was used to extract basic drugs from the acidic and neutral drugs in plasma. The acidic and the neutral drugs which exhibit a similar retention mechanism to that of the undissociated acidic compounds were adsorbed in the hydrophobic portion of the sorbent and eluted in the neutral fraction while the basic drugs were retained by the cation exchange interactions with the sorbent and eluted in the ammoniated fraction.

First, blank urine containing no drugs was treated using the SPE method. Figure 5a for the acidic and neutral elution con-

ditions (Elution 1) showed nothing eluting in the region of the acidic and neutral drugs in the standards. Figure 5b which depicts a blank urine using the basic elution conditions (Elution 2) also showed nothing eluting in the region of the acidic drugs. For the spiked urine samples, the neutral and acidic drugs were eluted in the neutral fraction (Figure 6a, Elution 1) since they were retained through hydrophobic interactions while the basic drugs, retained by the strong anion exchange functionalities of the sorbent, eluted separately in the basic fraction, as shown in Figure 6b, Elution 2. A small amount (< 10%) of the neutral/acidic drugs were also found in the basic fraction. A larger volume of methanol in the prior step could have been used to improve extraction efficiency.

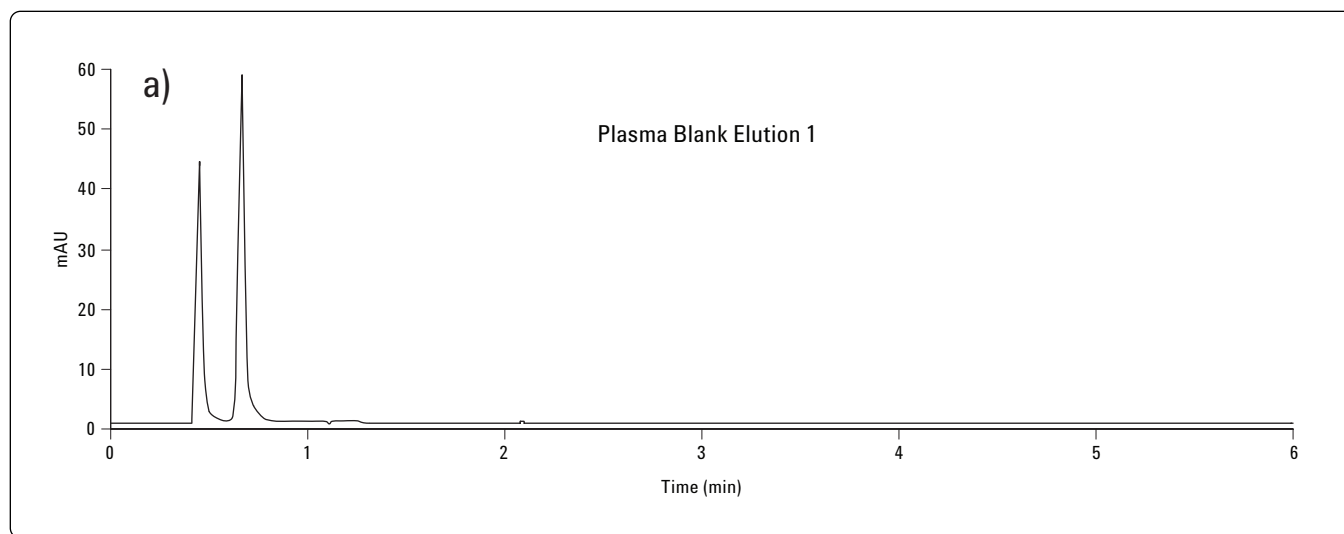


Figure 5a. Chromatograms of blank plasma sample from elution 1.

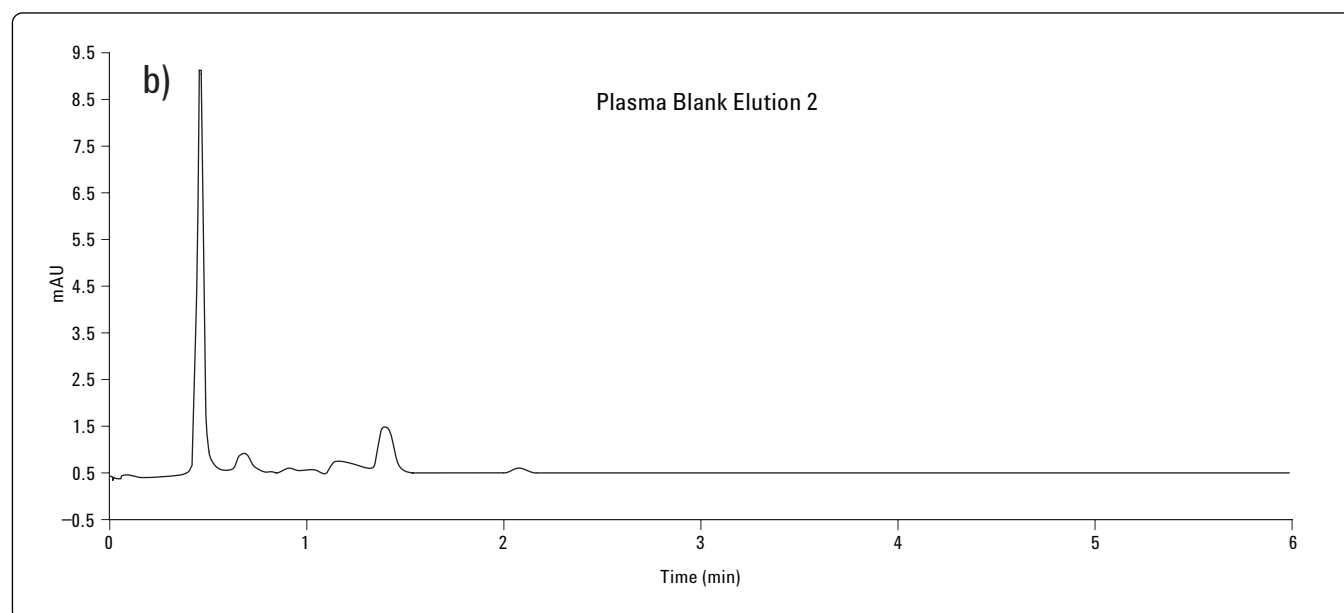


Figure 5b. Chromatograms of blank plasma sample from elution 2.

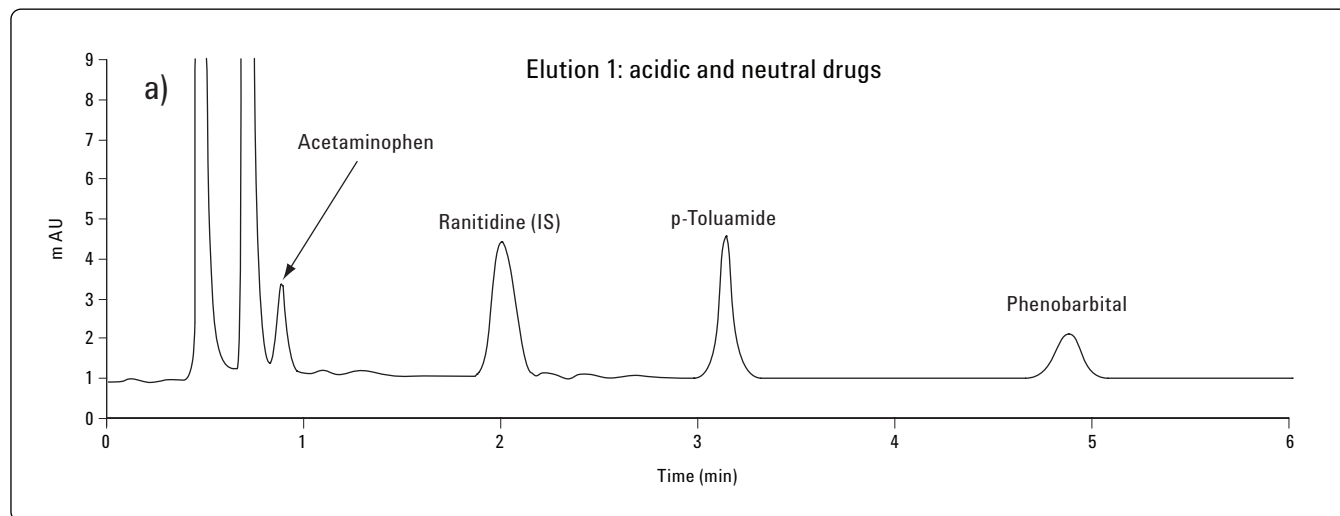


Figure 6a. Chromatograms of drugs in plasma, elution 1: 1. acetaminophen, 2. ranitidine (IS), 3. p-toluamide and 4. phenobarbital.

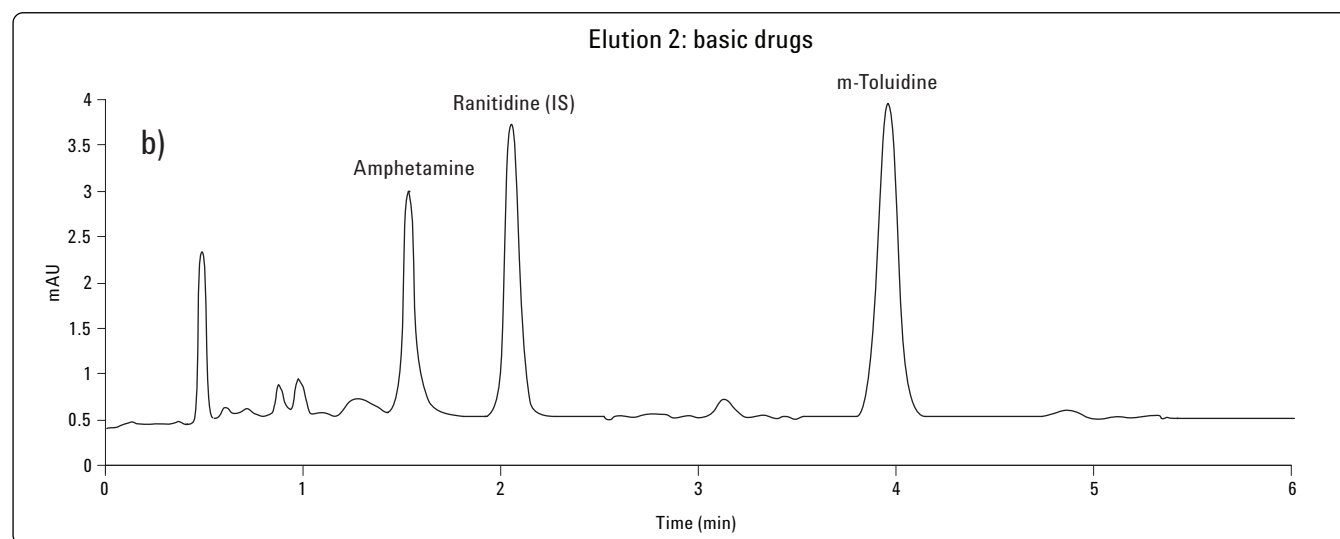


Figure 6b. Chromatograms of drugs in plasma, elution 2: 1. amphetamine, 2. ranitidine (IS), 3. m-toluidine.

Linearity, limits of detection and limits of quantification

The blank plasma was spiked with the analytes at five different concentrations and subjected to the SPE procedure in Figure 2. 50 μL of the internal standard was added. Each spiked plasma sample was prepared in triplicate. The analyte/IS peak area ratios were plotted against the corresponding concentrations. All the analytes were linear in the chosen concentration range (0–8 $\mu\text{g/mL}$) with $r^2 > 0.999$ as shown in Table 2.

Table 2. Linearity of the Method Employing SPE

Drugs	Concentration range (0–8 $\mu\text{g/mL}$)	
	Linear equation	Correlation coefficient (r^2)
Acetaminophen	0.099x	0.9999
Amphetamine	0.0853x	0.9991
p-Toluidine	0.138x	0.9990
m-Toluamide	0.1804x	0.9991
Phenobarbital	0.1526x	0.9991

The following equations 1 and 2 were used to calculate LOD and LOQ, where S_{yx} is the standard error of the regression line and b is the gradient.

Equation 1

$$LOD = \frac{3.3 \times S_{yx}}{b} \quad (1)$$

Equation 2

$$LOQ = \frac{10.0 \times S_{yx}}{b} \quad (2)$$

Table 3 shows the limits of detection and quantification determined for each analyte.

Table 3. LOD and LOQ for the Analytes

Drug	LOD ($\mu\text{g/mL}$)	LOQ ($\mu\text{g/mL}$)
Acetaminophen	0.39	0.85
Amphetamine	0.71	1.87
p-Toluamide	0.66	0.70
m-Toluidine	0.35	1.06
Phenobarbital	0.82	1.89

Recovery and reproducibility studies

Spiked plasma samples at three concentration levels: 0.5-, 2.5- and 5- $\mu\text{g/mL}$, corresponding to the lower, middle and upper limit of the linearity curve, were subjected to SPE clean-up. The analyte chromatographic peak areas obtained were compared to those obtained from standard solutions at the same concentration and the percentage extraction yield was calculated. To demonstrate reproducibility the samples were analyzed at the three mentioned concentration levels ($n = 6$). Good recoveries were obtained as indicated in Table 4.

Conclusions

The SPE procedure was successfully carried out on Agilent SampliQ SCX sorbents for the simultaneous extraction of acidic, basic and neutral drugs from plasma with high and reproducible recoveries ($> 80\%$). The LOD ranged from 0.39 to 0.84 $\mu\text{g/mL}$ for the drugs studied. The LOQ ranged from 0.71 to 1.89 $\mu\text{g/mL}$. This method can be applied to compounds that exhibit a diverse polarity and acidic, basic or neutral functionalities.

Table 4. Recoveries for the Drugs in the Study

Drugs	Class	Spike level ($\mu\text{g/mL}$)					
		0.5		2.5		5	
		Recovery	%RSD	Recovery	%RSD	Recovery	%RSD
Acetaminophen	Neutral	81.45	0.41	95.08	0.48	92.62	0.22
Amphetamine	Basic	83.16	0.62	88.83	1.09	86.64	0.28
p-Toluamide	Acidic	85.88	0.65	96.86	0.18	94.40	0.07
m-Toluidine	Basic	81.97	0.51	89.16	0.33	94.86	0.10
Phenobarbital	Acidic	85.49	0.39	90.13	0.25	80.48	0.21

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