Joint Pharmaceutical Analysis Group - Short Papers in Pharmaceutical Analysis

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Electrical impedance analysis of nutraceutical formulation stability in the frozen state

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Objectives Dielectric spectroscopy was used to examine the polarisation-relaxation response of a model nutraceutical, tea tree oil (TTO), alone and formulated with traditional excipients, when exposed to an electromagnetic field. The response provides a measure of mobility within a material, which is dependent on both structural and molecular properties (Craig 1995). TTO is a natural product obtained by steam distillation of leaves and twigs of the *Melaleuca alternifolia* tree—to conform to pharmaceutical grade requirements TTO must contain <5% cineole and >35% terpinen-4-ol. Traditionally, TTO is applied topically for localised treatment of bacterial, fungal, viral infections, etc., and is available commercially as essential oil, cream, ointment and lotion formulations (Reichling et al 2006).

Methods Pharmaceutical grade pure essential oil and polymer-stabilised oil-in-water emulsion formulations were analysed with Lyotherm2 (Biopharma Technology Limited), which assesses electrical impedance and differential thermal analysis (DTA) during cooling and warming of materials to indicate molecular mobility changes (possibly resulting from softening, relaxation, crystallisation, rearrangement or melting), which may be applicable to reduced temperature operations (i.e. freeze drying, cold storage and transport). Emulsions were prepared by vigorously mixing aqueous solutions of two different molecular weights (1 KDa and 10 KDa) of poly (ethylene glycol) [PEG] at three different concentrations (10.0, 1.0 and 0.1% w/v) with TTO in a 1:1 volumetric ratio. Cooling to >40 °C below the maximum impedance temperature was provided within the liquid nitrogen chamber and the samples were reheated to 20 °C (at 1.5 °C/min). The data was exported directly into MSExcel for analysis of the warming profile to determine the temperature of significant events which may be due to increases in molecular mobility and/or relaxation.

Results Following cooling to < −100 °C pure TTO (and TTO diluted (1% v/v) in dichloromethane) remains mobile and displays no discernable T_{onset} temperature point (a melt in the system); this implies that the pure essential oil would remain in a liquid state and during reduced temperature processing and storage. Conversely, TTO-PEG emulsions display distinct T_{onset} values between −1 and −5 °C (depending on molecular weight and concentration of PEG used), which implies the potential stability of these formulations at >−5 °C by virtue of their reduced molecular mobility. Interestingly, decreasing the concentration of PEG in TTO emulsions does not lead to a concurrent reduction in impedance indicating the stability of the formulation even at lower polymer levels.

Conclusions These results show the potential stability advantages for processing, storage and transport of lipophilic nutraceuticals, when formulated with low concentrations of emulsifying polymers. In conclusion it may be possible to maintain oil-base products at between 0 and −5 °C, thus conserving their bioactive properties, with the addition of commonly used inert, polymeric excipients such as PEG.

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Reichling, J., et al (2006) *Eur. J. Pharm. Biopharm*. **64**: 222–228

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The effect of anticoagulants on the distribution of chromium (VI) in blood fractions

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Objectives To investigate the influence of anticoagulants on the *in vitro* distribution pattern of hexavalent chromium in blood fractions. Many metallic implants used in orthopaedics are made of stainless steel or cobalt-chromium alloys which contain 18–30% chromium. Hexavalent chromium has been shown to be the predominant form of chromium released following *in vivo* and *in vitro* corrosion of these metal implants (Merritt & Brown 1995). Blood chromium levels may be elevated 50–250 times in patients with metal hip implants (Lhotka et al 2003). At physiological pH, hexavalent chromium exists predominantly as the chromate anion and as such can enter cells via non-specific anion channels. The anionic hexavalent chromium diffuses readily through the red blood cell (RBC) membrane and is bound by the haemoglobin probably after its rapid reduction to the cationic trivalent state within the RBC (Gray & Sterling 1950).

Methods Human blood withdrawn using various anticoagulants (sodium citrate, sodium heparin or EDTA) was spiked with clinically relevant concentrations of hexavalent chromium in the range 0–40 μg/L (NHS Research Ethics Ref No: 04/S0702/60). The blood after incubation for 45 min at room temperature was centrifuged and the plasma collected. The RBC portion was washed with saline. The concentration of chromium in plasma was determined using graphite furnace atomic absorption spectrometry (GFAAS) and that in RBC estimated by difference (i.e. Total Concentration – Plasma concentration).

Results The anticoagulants sodium citrate and sodium heparin showed similar results for the distribution of chromium in plasma and RBC (Table 1). A single factor analysis of variance showed a significant difference ($P < 0.05$) between the chromium level partitioned into the RBC fraction compared with that in plasma in blood stabilized by both sodium citrate and sodium heparin. However, a lower partitioning of chromium (VI) into RBC was observed in EDTA-stabilised blood compared with the other anticoagulants and there was no significant difference ($P > 0.05$) between the levels of chromium partitioned into RBC and the plasma fraction in EDTA stabilised blood. We propose that the chelating properties of EDTA influence the distribution of Cr in blood.

Conclusion The choice of anticoagulant employed to withdraw the blood affects the extent of partitioning of Cr (VI) into blood fractions.

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Table 1 The concentration of chromium (mean ± s.d, n = 3) in RBC and plasma of blood stabilized by the different anticoagulants

Spiked Blood concn (μg/L)	Sodium citrate		Sodium heparin		EDTA	
	RBC	Plasma	RBC	Plasma	RBC	Plasma
2	1.5 ± 0.3	0.5 ± 0.3	1.6 ± 0.3	0.4 ± 0.3	0.7 ± 0.2	1.3 ± 0.2
5	3.4 ± 0.2	1.6 ± 0.2	3.6 ± 0.8	1.6 ± 0.8	1.9 ± 0.3	3.1 ± 0.3
10	7.6 ± 0.5	2.4 ± 0.5	7.9 ± 1.2	2.1 ± 1.2	4.0 ± 0.6	6.0 ± 0.6
40	33.8 ± 0.3	6.3 ± 0.3	30.8 ± 4.0	9.2 ± 4.0	19.8 ± 2.1	20.2 ± 2.1

Data are expressed as mean ± s.d, n = 3.

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Lhotka, C., et al (2003) *J. Orthop. Res*. **21**: 189–195
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A new method for rapid, reliable and effective polymorph screening

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Objectives To develop a new, theoretically sound method for screening new active pharmaceutical ingredients (APIs) for possible polymorphism. Polymorphism can be a major issue in the development of new treatments, and current state of the art methods are not underpinned by any rigorous theory (Bernstein 2002).

Method A constrained crystallisation method was developed, which allows an enforcement of Ostwald's Rule of Stages. This well-established rule states that a crystallising system moves towards equilibrium (the most stable crystal polymorph) in stages. These stages correspond to metastable polymorphs. By reducing the number of degrees of freedom available to the crystallising system, it is possible to force the system to move to equilibrium in well-defined stages and thereby fully characterise its polymorphic behaviour.

Results Implementation of this new theory results in the ready isolation of all three polymorphs of paracetamol. This cannot be achieved using state of the art high-throughput methods, in which only forms I and II, but not form III, are found in 7776 crystallisation experiments (Peterson et al 2002). Typical experiment times for the new experimental protocol were of the order of minutes, with samples sizes of a milligram or less. This small sample size is important for early-stage screens, in which only a small amount of the development drug is usually available. Use of the method to investigate the API flufenamic acid (a COX-1 and COX-2 inhibitor) uncovers at least four polymorphs. Several further examples will be given.

Conclusions This new method may be generally applicable to screening of systems for possible polymorphism, which is an area of intense current interest in the pharmaceutical industry.

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An investigation into the use of X-ray powder diffraction (XRPD) for the analysis of intact tablets

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Objectives X-ray powder diffractometers are commonly used with reflection geometries for the analyses of APIs and ground dosage forms. Previous work has shown that the use of a transmission geometry can offer advantages of the reduction of preferred orientation effects and the analyses of intact tablets (Barnes & Busby 2005). The purpose of this study was to establish if there were limitations of the technique dependent on API loading, tablet thickness, tablet curvature, analysis time, excipient composition and tablet compression pressure. Crucially, experiments were performed to investigate whether the data obtained from intact tablets was of sufficient quality to allow the discrimination of solid-state forms. The powder patterns were assessed in terms of pattern completeness and resolution.

Methods A Phillips X'pert Pro (Phillips Analytical, Holland) with a Cu anode and an X'celerator detector was used; samples were analysed over the range of 3–40 °2θ. An elliptical mirror was used on the incident beam side and samples were presented between X-ray transparent foils. Tablets were analysed both as whole tablets and sectioned along the major axis. Several tablet types were used: tablets containing compound X were used for studies into the limitations of the technique and the discrimination of solid-state forms, tablets containing paracetamol with a range of excipients were used for the excipient composition studies and tablets containing compound Y were used for investigations into the influence of compression pressure. The monohydrate form of Compound X is under development for migraine and is currently entering phase 2 of the drug development process. Form C of compound Y is under development as an antiviral and is late in the drug development process.

Results Compound X could be identified in tablets containing 30 mg and 90 mg of drug substance that were presented as whole tablets and sections. The signal:noise ratio of the patterns was better in the sectioned tablets and the whole tablets with the lower drug loading of 30 mg. Interestingly, for the same section, a small difference in signal was obtained depending on the tablet curvature, which is likely due to scattering effects. Varying the data collection time for sectioned tablets containing 90 mg of compound X showed that complete patterns with good resolution could be obtained after 2 h. Monohydrate and trihydrate forms of compound X were easily discriminated from sectioned tablets of a 90-mg strength and whole tablets of a 30-mg strength. Paracetamol tablets containing a range of excipients produced patterns of varying quality. Tablets of compound Y processed with compression pressures of 14 or 26 kN did not produce any differences in the diffraction pattern.

Conclusions The results of this study show that the data obtained from intact tablets is dependent on the drug loading, tablet thickness, tablet curvature and excipient composition. Further, different solid state forms of compound X can be identified in intact tablets. Good quality data was obtained from tablets containing compound X within 2 h and tablets containing compound Y did not appear to be affected by compression pressure within the ranges studied.

Barnes, C. A., Busby, D. J. (2005) *J. Pharm. Pharmacol*. **57**: S82

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Microemulsion high performance liquid chromatography method for the determination of terbutaline in urine samples

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Objectives Microemulsion High Performance Liquid Chromatography has previously been used to separate drugs and their related impurities in pharmaceutical formulations (Marsh et al 2004; Altria et al 2006). However, very few studies have used microemulsion HPLC to separate drugs in biological fluids (El-Sherbiny et al 2003). Furthermore, none of these studies have validated HPLC methods for separation in biological fluids. This report describes the development and validation of a microemulsion HPLC method for the determination of terbutaline in urine samples. This method used the isocratic oil-in-water microemulsion to separate the terbutaline from the endogenous urine components. The urinary assay was performed in accordance with FDA and ICH guidelines for the validation of bioanalytical samples.

Methods The samples were injected into C18 Spherisorb (250 mm × 4.6 mm × 5 μm) columns at 42 °C using a constant flow rate of 1 mL/min. The mobile phase was 91.5% aqueous orthophosphate buffer (adjusted to pH 3 with ortho phosphoric acid), 0.5% ethyl acetate, 1.5% Brij35, 2.5% 1-butanole, 4% octane sulfonic acid, all w/w. The terbutaline peak was detected by fluorescence using excitation and emission wavelengths of 265 and 313 nm, respectively. A solid phase extraction (SPE) using Oasis HLB was used to extract the terbutaline and bamethan (internal standard) from spiked urine samples.

Results The extraction recovery of terbutaline from urine samples was >90% and the calibration curve was linear ($r^2 = 0.99$). The method had an inter-day precision (CV %) of <2.62 and good accuracy. The limit of detection (LOD) was 35 μg/L. The influence of the composition of the microemulsion system was also studied and the method was found to be robust with respect to some changes, but not to others.

Conclusion The method described in this study offered faster analysis times and equivalent sensitivity to conventional HPLC modes. This demonstrates potential utility of these methods for the determination of inhaled terbutaline in urine samples.

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Ferulic acid assay and its relevant use in quality assurance of medicinal herbs

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Objectives Ferulic acid (FA) is one of the bioactive compounds in some functional plants and usually chosen as a chemical marker for quality assessment of certain medicinal materials. However, its accurate quantification in plant samples is always problematic. This study is to develop a rapid and accurate analytical method to determine the reliable FA content in plants samples and determine a suitable FA level for the quality assurance of relevant medicinal herbs.

Methods An accurately weighed quantity of sample powder was extracted in methanol–formic acid (95:5) and methanol–2% sodium hydrogen carbonate in water (95:5) by sonication for the assay of free FA and total available FA, respectively. The extracts were filtered for high-performance liquid chromatography (HPLC) analysis. HPLC analysis was conducted on an Alltima C_{18} column (5 μm, 250 mm × 4.6 mm) with a mixture of 1.0% acetic acid in water (A) and acetonitrile (B) as mobile phase using a gradient program of 19% B in 0–18 min, 19–100% B in 18–60 min. The flow rate was 1.0 mL/min and the column temperature was maintained at 30 °C. The DAD detector was set at 320 nm (Lu et al 2005). FA was quantified in herb using an external standard calibration method with a reference marker.

Results Altogether 14 herb samples under 6 herb names and 7 plant species were quantitatively analyzed for free FA and total available FA (Table 1). The amount of total available FA and free FA and their ratios in the 14 tested samples were remarkably different with the ratio of 2.94 ± 1.95 (mean ± SD, n = 14). It indicated that the amount of total available FA was generally 2 times more than that of free FA in these samples.

Conclusions Free FA refers to the natural abundance and is considered as a characteristic of herbal species. Contrarily, total FA indicates the sum of free and those hydrolysed from conjugates which represents the available amount of FA for therapeutic effect in Chinese medicine practice. Their ratios are significantly different among plant species. In our study, we found that total FA is a better indicator for the quality assessment of medicinal herb as well as fulfilling the purpose of screening alternative botanical resources of FA.

Table 1 Content of free FA and total available FA in samples

No.	Herb name[a]	Free FA[b]	Total FA[b]	Ratio[c]
1	Chinese Angelica	0.177	1.06	5.97
2	Chinese Angelica	0.387	1.01	2.60
3	Chinese Angelica	0.111	0.914	8.23
4	Chinese Angelica	0.387	1.22	3.15
5	Japanese Angelica	0.047	0.049	1.04
6	Japanese Angelica	0.106	0.123	1.16
7	Korean Angelica	0.151	0.233	1.54
8	Korean Angelica	0.070	0.152	2.17
9	Cnidium Rhizome	0.580	1.28	2.21
10	Cnidium Rhizome	0.504	0.703	1.39
11	Lovage Root	0.064	0.182	2.84
12	Lovage Root	0.058	0.176	3.03
13	Chinese Lovage	0.189	0.523	2.77
14	Chinese Lovage	0.282	0.869	3.08

[a]The plant sources of these herbs were the roots of *Angelica sinensis* (Oliv.) Diels (1–4) collected from China, *A. acutiloba* Kitag (5), *A. acutiloba* Kitag. var. *sugiyamae* Hikino (6) from Japan, *A. gigas* Nakai (7, 8) from Korea, the rhizome of *Cnidium officinale* Makino (9, 10) from Japan, the roots of *Levisticum officinale* Koch (11, 12) from Germany, the rhizome and roots of *Ligusticum acuminatum* Franch (13) and *L. jeholense* (Nakai et Kitag.) Nakai et Kitag (14) from China. [b]The value was mean of two replicate analyses (mg/g) and expressed in three significant figures. [c]The ratio on the amount of total available FA to free FA in the samples.

Lu, G. H. et al (2005) *J. Chromatogr. A* **1068**: 209–219

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Sensitive and specific liquid chromatography-tandem mass spectrometric (LC-MS/MS) method for estimation of digoxin in human serum

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Objective Digoxin is a potent cardiac glycoside widely used for the treatment of congestive heart failure. It is a narrow therapeutic index drug available in different strengths. Digoxin therapy requires strict monitoring of blood levels. There are various methods reported in the literature for estimation of digoxin in biological fluids—viz. HPLC with UV or fluorescence detection, RIA and LC-MS/MS. However, they have limitations either in terms of sensitivity or specificity. The objective of this study was to develop a specific and sensitive LC-MS/MS method for monitoring digoxin levels in human serum that can be utilized for assessment of pharmacokinetics/bioavailability/bioequivalence of digoxin formulations.

Methods In the present method digoxin and the internal standard (ISTD), digitoxin, were extracted from serum (450 μL) employing liquid-liquid extraction using mixture of dichloromethane and tert-butyl methyl ether (25:75; 3 mL). The organic extract was evaporated to dryness under nitrogen using TurboVap LV. The residue was reconstituted in mixture of ammonium formate buffer (10 mM, pH 3.5) and acetonitrile (50:50 v/v) and injected onto the system (Sciex API4000 LC-MS/MS with electrospray ionization (ESI) turboionspray inlet). Digoxin and ISTD were resolved on Hypurity Advance C18 (5.0 μ, 4.6 × 50 mm) column using a mobile phase consisting mixture of ammonium formate buffer (10 mM, pH 3.5):acetonitrile (60:40) as mobile phase flowing at the rate of 0.3 mL/min (analysis time = 8.0 min). Digoxin and ISTD (ammonium adducts) were monitored in the positive ion multiple reaction monitoring (MRM) mode at transitions of 798.5→651.5 (m/z) and 782.8→635.5 (m/z), respectively.

Results The method employed a simple and single step extraction, enabling faster sample processing. Digoxin (RT = 3.50 ± 0.5 min) and ISTD (RT = 6.20 ± 0.5 min) were well resolved. The method is specific and sensitive requiring only 450 μL of sample with LOQ of 0.0475 ng/mL. The method was validated and the validation parameters are summarized below.

Table 1 Validation parameters for the proposed LC-MS/MS method for estimation of digoxin in human serum

Validation parameter	Results (digoxin)
Sensitivity (ng/mL) LOQ	0.0475
Linearity range (ng/mL)	0.0475–7.916
Accuracy (%)	
Within-batch	99.04–115.62
Between-batch	101.89–108.56
Ruggedness (different analyst)	99.07–113.47
Precision (%CV)	
Within-batch	1.61–8.01
Between-batch	4.57–8.75
Ruggedness (different analyst)	2.10–5.85
Recovery (%)	80.17 (79.02 – ISTD)
Dilution integrity (Accuracy %)	
(1:2 dilution)	108.47%
(1:4 dilution)	98.64%
Re-injection reproducibility (%)	87.45 (LQC); 97.19 (HQC)
Stock solution stability	
Short-term (6.0 h)	92.90 (94.84 – ISTD)
Long-term (17 days)	99.83 (104.49 – ISTD)
Serum sample stability	
Freeze-thaw (3 cycles)	94.90 (LQC); 103.24 (HQC)
Bench-top (8.0 h)	96.30 (LQC); 97.95 (HQC)
Autosampler (44.5 h)	89.79 (LQC); 100.98 (HQC)
Long-term (18 days)	97.59 (LQC); 110.09 (HQC)

Conclusion The present LC-MS/MS method offers advantages, such as smaller sample volume, simple sample cleanup and very high sensitivity (LOQ = 0.0475 ng/mL). The method was found to be simple, sensitive, precise, accurate and specific for estimation of digoxin in human serum and was successfully employed for bioequivalence study of digoxin formulations.

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A rapid and sensitive method for simultaneous determination of carboxylate and lactone forms of irinotecan HCl by ultraviolet high-performance liquid chromatography

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Objectives Irinotecan (CPT-11) is a semisynthetic derivative of camptothecin, an alkaloid extract from plants such as Chinese Camptotheca acuminate. Camptothecin derivatives are known to have an anti-tumor activity. CPT-11 and other camptothecin analogues are sensitive to a pH-dependent reversible conversion between a pharmacologically active lactone form and its inactive, lactone ring-opened, carboxylate form. In all of the methods developed for CPT-11 analysis that are published until now, the detection method is established on the fluorescence detector, and there is no method based on UV detector. As ultraviolet detection of the compounds is more common than the fluorescence detection, in this study we developed a new simple HPLC method for determination of the CPT-11 in vitro.

Methods CPT-11 as trihydrate chloride salt was provided by Aurisco, China. HPLC-grade acetonitrile, analytical grade triethylamine (TEA), acetic acid, citric acid, boric acid, sodium hydroxide were purchased from Merck, Germany. The isocratic system utilized consisted of a quaternary HPLC pump (G1311A, Agilent technologies, USA) and an autosampler system (G1329A, Agilent technologies, USA) fitted with a 20 μL sample loop, and a diodarray detector (G1315B Agilent technologies, USA). Separation of compounds was achieved using a Nucleosil RP-18 (5 μm, 250 × 4.0 mm) analytical column protected by a C_{18} Nucleosil precolumn

(5 μm, 4.0 × 4.0 mm). The mobile phase is triethylammonium acetate buffer:acetonitrile and detection carryout under the 254 nm wavelength. A study of lactone-carboxylate structural conversion of CPT-11 under the different type of vehicles was carried out to determine the optimal media in which the structural conversion was minimal. All validation runs were performed on three consecutive days and all samples used for validation were prepared as standard samples. Three different concentrations (0.1, 5 and 10 μg/mL) of both forms of CPT-11 were investigated for recovery. The concentration of each form in each sample was determined using its standard curve. Then, the percent ratios of measured concentration to known added concentration were calculated in each case. Quality control samples were prepared from weighing independent of those used for preparing calibration curves. Within-day and between-day precision and the mean accuracy were determined by analysis of both lactone and carboxylate forms at 0.1, 1, 2, 5 and 10 μg/mL triplicate on a single day and on 3 consecutive days respectively. The quality control samples were prepared on the day of analysis in the same way as calibration standards. During each analytical run, QC samples were included and processed as the calibration and unknown samples.

Results Representative chromatograms for the two forms of CPT-11 are shown in Figure 1. Under the chromatographic conditions, the retention times for lactone form of CPT-11 was 4.8 ± 0.1 and for carboxylate form was 2.4 ± 0.2 (n = 40). The mean correlation coefficients (r) for the daily calibration curves were all >0.999 (n = 5) and the within- and between-run CVs of the response factors for each concentration assayed were below 10%. In summary, we reported on a simple and reliable HPLC method for simultaneous measurement of lactone and carboxylate forms of CPT-11 in vitro.

Conclusions In conclusion, we have demonstrated a simple and reliable HPLC method for analysis of the irinotecan HCl in both lactone and carboxylate forms by UV detection.

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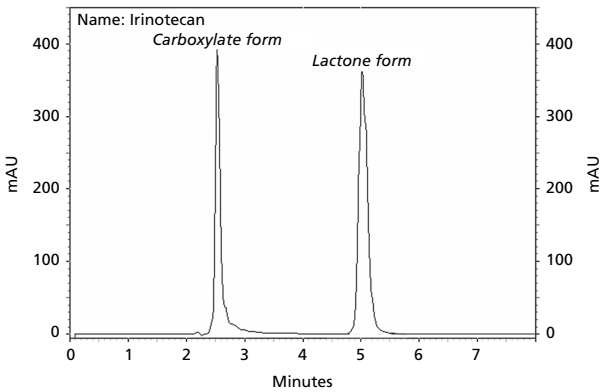

Figure 1 Chromatogram of irinotecan HCl (lactone and carboxylate forms), 50 μg/mL.