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Short communication

Comparison of plasma sample purification by manual liquid–liquid extraction, automated 96-well liquid–liquid extraction and automated 96-well solid-phase extraction for analysis by high-performance liquid chromatography with tandem mass spectrometry

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Abstract

Three extraction procedures were developed for the quantitative determination of a carboxylic acid containing analyte (**I**) in human plasma by high-performance liquid chromatography (HPLC) with negative ion electrospray tandem mass spectrometry (MS–MS). The first procedure was based on the manual liquid–liquid extraction (LLE) of the acidified plasma samples with methyl *tert*.-butyl ether. The second procedure was based on the automation of the manual LLE procedure using 96-well collection plates and a robotic liquid handling system. The third approach was based on automated solid-phase extraction (SPE) using 96-well SPE plates and a robotic liquid handling system. A lower limit of quantitation of 50 pg/ml was achieved using all three extraction procedures. The total time required to prepare calibration curve standards, aliquot the standards and plasma samples, and process a total of 96 standards and samples by manual LLE was three-times longer than the time required for 96-well SPE or 96-well LLE (4 h, 50 min vs. 1 h, 43 min). Even more importantly, the time the bioanalyst physically spent on the 96-well LLE or 96-well SPE procedure was only a small fraction of the time spent on the manual LLE procedure (<10 min vs. 4 h, 10 min). It should be noted that the 96-well SPE procedure incorporated the two steps of evaporation of the eluates to dryness and subsequent reconstitution of the dried extract. The total time required for the 96-well SPE could be reduced by 50% if the eluates were injected directly, eliminating the drying and reconstitution steps, which is achievable when sensitivity is less of an issue.

Keywords: Plasma sample purification

1. Introduction

The speed of analysis of biological fluids for drugs and metabolites has significantly increased thanks to the use of high-performance liquid chromatography (HPLC) coupled with electrospray or atmospheric pressure chemical ionization (APCI) tandem mass spectrometry (MS–MS). It is now realized that the sample preparation (extraction) steps of LC–MS–MS bioanalytical methods have become the bottleneck which is slowing further improvement of overall speed of sample analysis. Two techniques have been

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explored to alleviate the sample preparation bottleneck. The first technique is based on direct injection of biological samples into an LC–MS–MS system that incorporates a specialized column and a switching valve [1–4]. This on-line sample preparation technique is simple and straightforward, and can be implemented using existing laboratory equipment with no additional capital outlays. However, it should be noted that when the one-column approach of this direct injection technique is used, which provides little chromatographic separation, the LC–MS–MS method may not be specific/selective to the analyte (drug) when analyzing samples that contain metabolites or a prodrug in addition to the drug [5]. The second technique is based on the use of 96-well solid-phase extraction (SPE) plates in conjunction with a robotic liquid handling system [6–10]. We now report on the application of the 96-well automation approach to liquid–liquid extraction (LLE) of plasma samples. We compare the 96-well LLE not only to manual LLE but also to 96-well SPE.

2. Experimental

2.1. Chemicals and reagents

Compound **I**, the analyte (Fig. 1), is a product of Bristol-Myers Squibb Pharmaceutical Research Institute. Compound **II**, the internal standard (Fig. 1), is a pentadeuterated stable isotope analog of **I**. Acetonitrile (HPLC-grade), formic acid (98%) and methyl *tert*.-butyl ether (HPLC-grade) were purchased from EM Science (Gibbstown, NJ, USA). Laboratory-deionized water, further purified with a Milli-Q water purifying system (Millipore, Bedford, MA, USA), was used. Drug-free human plasma was purchased from Biological Specialty (Colmar, PA, USA). A 1 m*M* formic acid solution was prepared by dissolving 0.043 ml of formic acid in 1000 ml of the Milli-Q water. Reconstitution solution was prepared by mixing water with acetonitrile in a 60:40 ratio and adding enough formic acid to obtain a 1 m*M* solution.

2.2. Equipment

LC–MS–MS analysis was performed with a Finnigan (San Jose, CA, USA) TSQ-7000 triple quadrupole mass spectrometer equipped with an API 1 electrospray interface and Interactive Chemical Information System (ICIS). A Hewlett-Packard HP1090L HPLC system (Hewlett-Packard, Palo Alto, CA, USA), equipped with an automatic autosampler, was used for procedure A. A Gilson 233XL autosampler (Gilson, Middleton, WI, USA), capable of injecting from 96-well plates, was used for procedures B and C. The HPLC analytical column was a BDS Hypersil C_8, 5 μm particle size, 50×2.0 mm, from Keystone (Bellafonte, PA, USA).

A robotic liquid handling system, Multiprobe I,

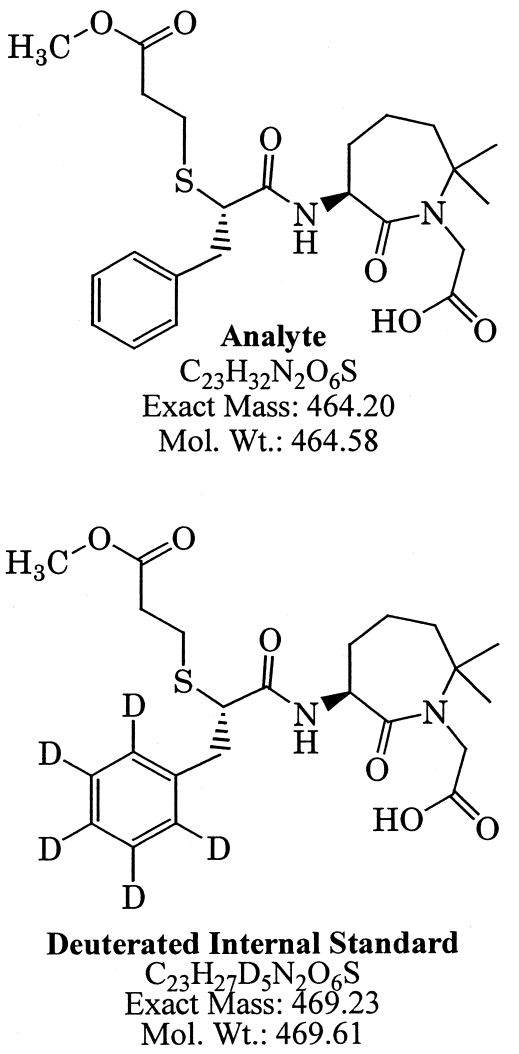

Fig. 1. Chemical structures of the analyte and the stable isotope labeled internal standard.

from Packard was used (Downers Grove, IL, USA). The 96-well SPE extraction plates, C_{18}, were from 3M (St. Paul, MN, USA). The 96 deep-well collection plates as well as the cover mats were from Beckman (Fullerton, CA, USA). A Savant evaporator, Model Speed Vac AES-2000, was used for evaporation of the extracts (Farmingdale, NY, USA). The Megafuge centrifuge from Baxter Scientific (McGaw Park, IL, USA) and the IEC centrifuge from International Equipment (Needham Heights, MA, USA) were used. The shaker used was an Eberbach two-speed shaker, purchased from Baxter Scientific (Ann Arbor, MI, USA).

2.3. Chromatographic and mass spectrometric conditions

Isocratic chromatography was employed using a mobile phase consisting of 1 m*M* formic acid–acetonitrile (60:40). The analytical column was maintained at 40°C and the flow-rate was 0.3 ml/min. The injection volume was 10 μl for manual LLE and 20 μl for automated LLE or SPE. The total run time was 2 min. The mass spectrometer was operated in the negative ion electrospray mode. The spray voltage was set to 4.5 kV and the heated capillary temperature was set to 250°C. The argon collision gas pressure was set to 2.5 mTorr (1 Torr= 133.322 Pa). The collision energy was set at 15 eV. The half-height mass peak width was 1 mass unit for Q1 and 0.7 mass unit for Q3. The mass monitoring window was 0.6 mass unit and the scan rate was 0.5 s/scan. The samples were analyzed via selected reaction monitoring (SRM) employing the transition of the $[M-H]^-$ precursor ion to product ion: m/z 463 to m/z 377 for **I** (analyte) and m/z 468 to m/z 382 for **II** (internal standard).

2.4. Standard and quality control (QC) preparations

The standard curve was prepared by spiking specified amounts of **I** into specified volumes of drug-free human plasma. The standard curve range in human plasma was 50 to 10 000 pg/ml. Four levels of QC samples were also prepared by spiking **I**, from a separate stock solution, into drug-free plasma. Three QC levels were in the first quartile, near the mid-point, and in the fourth quartile of the curve, respectively. The fourth QC sample, known as the dilution QC, had a concentration above the upper limit of the standard curve. The standards and QC samples used in procedure A were prepared manually; those used in procedures B and C were prepared using the robotic liquid handling system.

2.5. Extraction procedures

In procedure A, where manual LLE was used, an aliquot of 0.50-ml of each human plasma standard and QC sample containing **I** was transferred into a 100×16 mm screw cap glass tube. The dilution QC was diluted by a factor of 10 by combining 0.05 ml of QC with 0.45 ml of blank plasma. Then 0.01 ml of internal standard solution (to obtain 5000 pg/ml of human plasma) and 0.5 ml of 0.1 *M* hydrochloric acid solution were added and vortexed. To each acidified plasma sample, methyl *tert*.-butyl ether (3 ml) was added. The tubes were capped and shaken for 10 min. The aqueous and the ether layers were separated by centrifugation for 10 min. The tubes were placed in a dry ice/acetone bath to freeze the aqueous layer. After uncapping, the organic layer from each tube was poured into a 100×13 mm test tube; the ether was then removed by evaporation for 40 min in a Savant evaporator. Each dried extract was reconstituted by dissolving in 100 μl of the reconstitution solution (prepared as in Section 2.1). Each sample was transferred to an autosampler vial, which was capped and placed on the autosampler for injection of 10 μl of each sample.

In procedure B, where 96-well automated LLE was used, 0.10-ml portions of human plasma standards and QC samples containing **I** were transferred, using the robotic liquid handling system, into separate 100×13 mm glass tubes contained in a rack. The dilution QC was diluted by a factor of 10 by combining 0.01 ml of QC with 0.09 ml of blank. Then 0.05 ml of internal standard solution (to obtain 5000 pg/ml of human plasma) and 0.1 ml of 0.1 *M* hydrochloric acid solution were added using the robotic system. To each acidified plasma sample, methyl *tert*.-butyl ether (0.6 ml) was added using the robotic system. The rack containing the tubes was vortexed manually for 2 min. The aqueous and the ether layers were separated by centrifugation for 3 min. The organic layers from the tubes were trans-

ferred to a deep 96-well collection plate using the robotic system. The extracts in the 96-well plate were evaporated to dryness using the Savant evaporator. The dried extracts in the 96-well plate were dissolved by adding 50 μl of the reconstitution solution using the robotic system. The 96-well plate was sealed with a cover mat and placed on the autosampler, ready for injection of 20 μl from each sample.

In procedure C, where automated SPE was used, all the steps were automated using the robotic system, unless otherwise indicated. A 96-well C_{18} SPE plate was conditioned by passing 200 μl of methanol followed by 200 μl of 1 m*M* formic acid–methanol (95:5). An aliquot of 0.10-ml of each human plasma standard and QC sample containing **I** was transferred to the 96-well SPE plate. The dilution QC was diluted by a factor of 10 by combining 0.01 ml of QC with 0.09 ml of blank. Then 0.05 ml of internal standard solution (to obtain 5000 pg/ml of human plasma) and 250 μl of 0.1 *M* ammonium acetate buffer (pH 3.5) were added. Vacuum was applied for 0.5 min. Samples were rinsed by applying 400 μl of 1 m*M* formic acid–methanol (95:5). The waste tray was then removed manually and replaced with a collection plate. Elution was performed with 200 μl of acetonitrile. The collection plate was manually placed in a Savant evaporator to evaporate the eluates to dryness. The collection plate was placed back on the robotic system for reconstitution with 50 μl of water–acetonitrile (60:40) with 1 m*M* formic acid. The 96-well plate was sealed with a cover mat, vortexed and placed on the autosampler, ready for injection of 20 μl from each sample.

3. Results and discussion

Table 1 summarizes the time taken by the different steps for processing 96 samples using the three extraction procedures. The total time required for manual LLE was three-times longer than that required for automated LLE or automated SPE (4 h, 50 min for manual LLE vs. 1 h, 43 min for automated LLE and 1 h, 41 min for automated SPE). Even more importantly, the amount of time physically spent by the analyst using automated LLE or automated SPE was only a small fraction of the time spent using manual LLE (<10 min vs. 4 h, 10 min). Unlike automated SPE, automated LLE included labeling of a set of 96 tubes and a relatively long step to transfer the methyl *tert*.-butyl layers to a 96-well collection plate. However, the evaporation step of the automated SPE procedure took longer than that of the automated LLE, as the methyl *tert*.-butyl ether extracts evaporated faster than the acetonitrile eluates. Therefore, the total length of time required for the two procedures was about the same (1 h, 43 min for LLE vs. 1 h, 41 min for SPE). For the automated SPE procedure, the intervention by the analyst included removal of the waste collection tray and replacement with the 96-well collection plate, placement of the collection plate in the evaporator for drying, replacement of the collection plate on the robot deck for the automatic dispensing of the reconstitution solution, vortexing the plate, sealing of the plate with a cover mat and finally, placement on the HPLC autosampler. For the automated LLE procedure, the intervention by the analyst included the removal of the 96 tubes from the robot deck for vortexing and centrifuging and their sub-

Table 1
Time analysis of the three extraction procedures

	Manual LLE	Automated LLE	Automated SPE
Standard curve preparation	25 min	8 min	8 min
Labeling of tubes	Two sets of tubes, one set of autosampler vials: 15 min	One set of tubes: 5 min	None
Sample transfers and extraction	180 min	65 min	43 min
Drying	40 min	20 min	45 min
Reconstitution and transfer	30 min	5 min	5 min
Total time	4 h, 50 min	1 h, 43 min	1 h, 41 min
Analyst time	4 h, 10 min	<10 min	<5 min

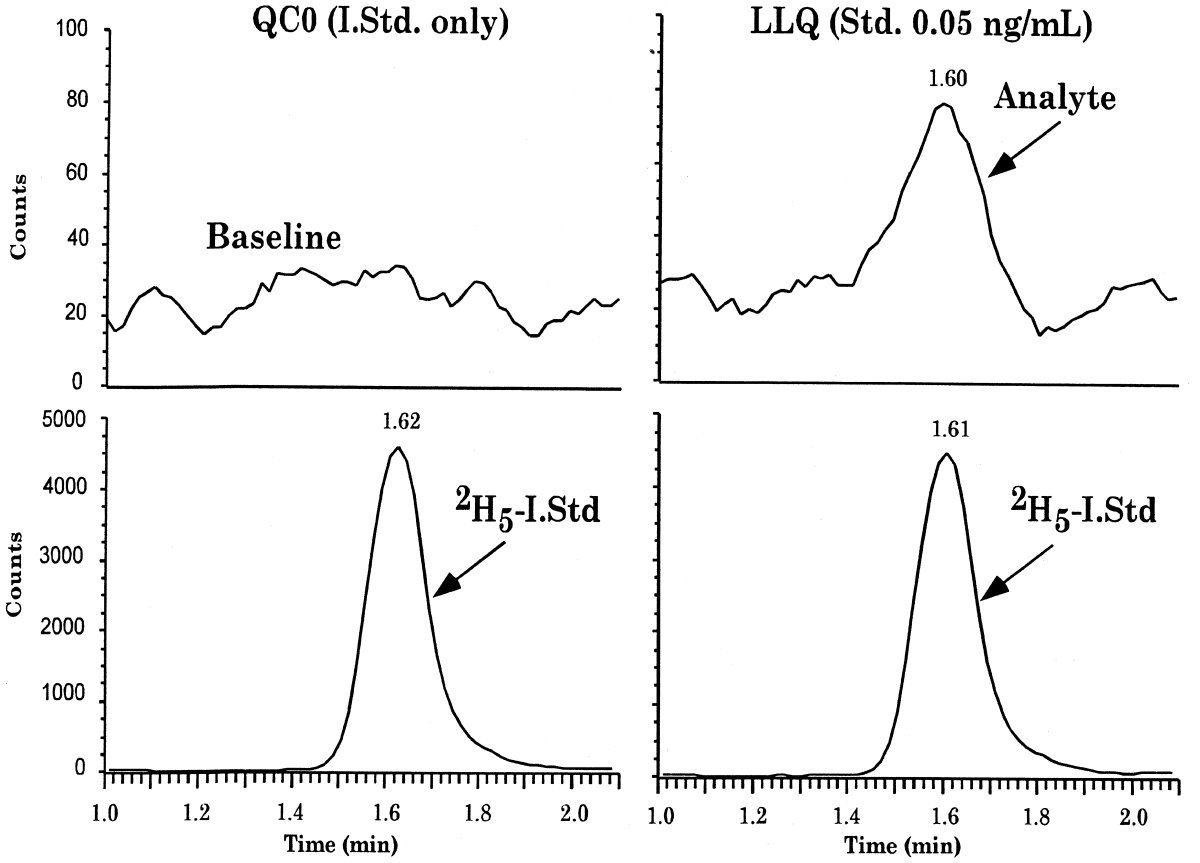

Fig. 2. SRM ion chromatograms of a plasma sample containing internal standard only at 5.0 ng/ml (QC0) and of a plasma sample containing the analyte at 50 pg/ml and the internal standard at 5.0 ng/ml (STD 0.05 ng/ml) following manual LLE. Retention time of the analyte and internal standard=1.60/1.61 min.

sequent replacement, removal of the collection plate containing the extracts for placement in the evaporator for drying, the replacement of the collection plate onto the robot deck for the automatic dispensing of the reconstitution solution, vortexing of the plate, sealing of the plate with a cover mat and finally, placing the plate on the HPLC autosampler. The LLE procedure included the additional step of labeling a set of 96 tubes used for the actual extraction. Thus, the total analyst intervention time was approximately 5 min for automated SPE and 10 min for automated LLE. It should be noted the total time required for automated SPE would be reduced to 53 min if the SPE eluates were injected directly from the plate without prior evaporation to dryness and reconstitution. This approach is feasible when sensitivity of the method is not an issue and hence the concentration step of evaporation is not required.

Operationally, manual LLE, which requires labelings and cappings, is time consuming and labor intensive, compared to automated LLE and automated SPE. Of the latter two, automated SPE is the less labor intensive and has the potential to be significantly less time consuming when the evaporation step of SPE eluates can be eliminated. Some analysts may still prefer automated LLE over automated SPE because the former, depending on the extraction solvent used, may give cleaner extracts as evidenced by less tendency for pressure buildup in the HPLC column as more samples are injected. In

Table 2
Accuracy and precision obtained with the three extraction procedures

Extraction technique	Spiked concentration (ng/ml)	Grand mean (ng/ml)	Deviation (%)	Inter-day (RSD, %)	Intra-day (RSD, %)
Manual LLE	0.236	0.246	4.1	12	7.6
	4.72	4.82	2.1	9.3	2.7
	7.87	7.71	−2.1	8.8	3.1
	78.7	83.6	6.2	5.0	2.4
Automated LLE	0.240	0.239	−0.32	2.6	7.6
	4.79	4.76	−0.68	5.0	6.0
	7.99	7.64	−4.3	8.4	7.1
	79.9	83.2	4.1	3.4	5.1
Automated SPE	0.240	0.259	7.8	0.52	9.1
	4.79	4.46	−6.8	4.3	3.1
	7.99	7.57	−5.2	0.87	9.6
	79.9	78.3	2.0	0.31	5.8

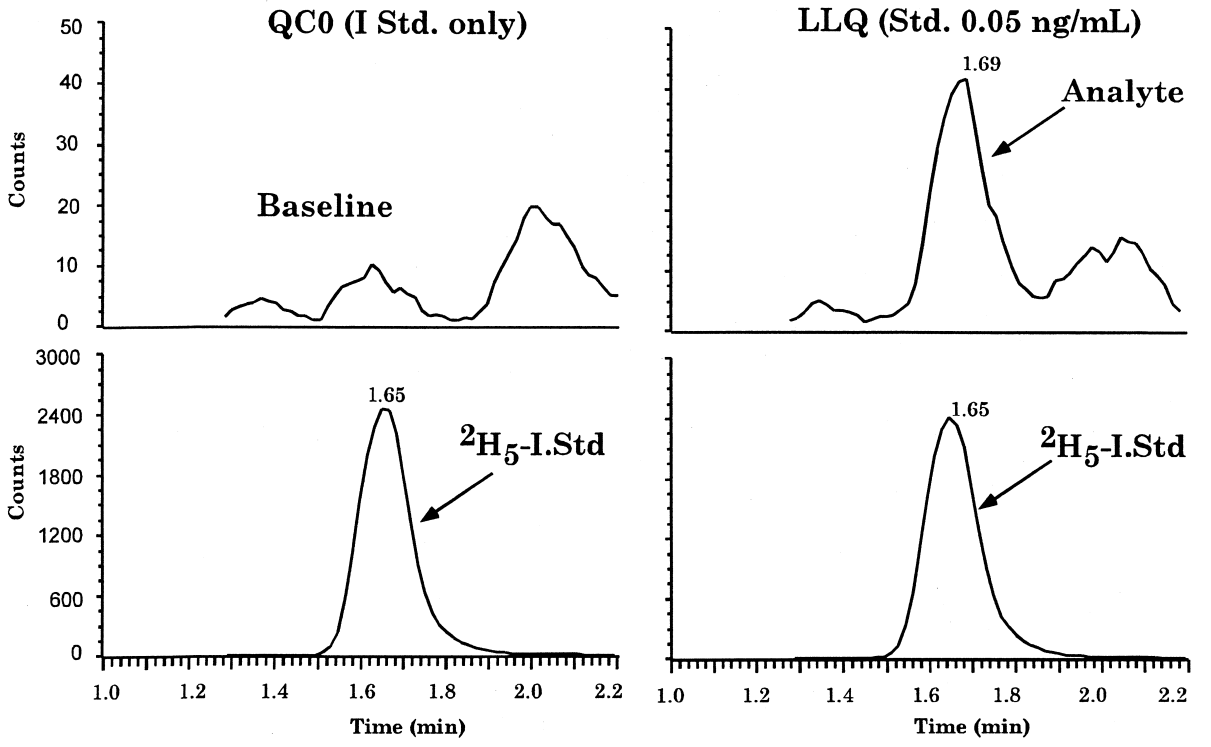

Fig. 3. SRM ion chromatograms of a plasma sample containing internal standard only at 5.0 ng/ml (QC0) and of a plasma sample containing the analyte at 50 pg/ml and the internal standard at 5.0 ng/ml (STD 0.05 ng/ml) following automated LLE. Retention time of the analyte and internal standard=1.69/1.65 min.

addition, with use of the LLE procedure, the analyst avoids a common problem associated with SPE, namely, blockage of the extraction column due to clots in the biological fluids. On the other hand, it should be noted that relatively polar analytes that are difficult to isolate from the biological samples by LLE can be isolated by SPE. The chance for the analyst exposure to biohazardous biological samples was equally minimized using automated LLE or SPE.

The comparison of the performance of the LC–MS–MS method using the three extraction procedures is shown in Table 2, which summarizes the accuracy and precision data obtained from three runs on three different days. For each extraction procedure, four levels of QC samples were analyzed in five replicates in each run. The accuracy was excellent as the deviations from nominal concentrations were within 7.8% for all concentrations and all three procedures. The precision was also excellent as the intra- and inter-day relative standard deviation (RSD) values were within 12%. Figs. 2–4 compare, for each extraction procedure, the SRM chromatograms obtained with plasma samples spiked with the internal standard only (QC0) against those obtained from plasma samples spiked with the internal standard and the analyte at the lower limit of quantitation (LLQ), 50 pg/ml. The LLQ samples gave a good, quantifiable analyte peak while the QC0 samples gave little or no peak at the retention time of the

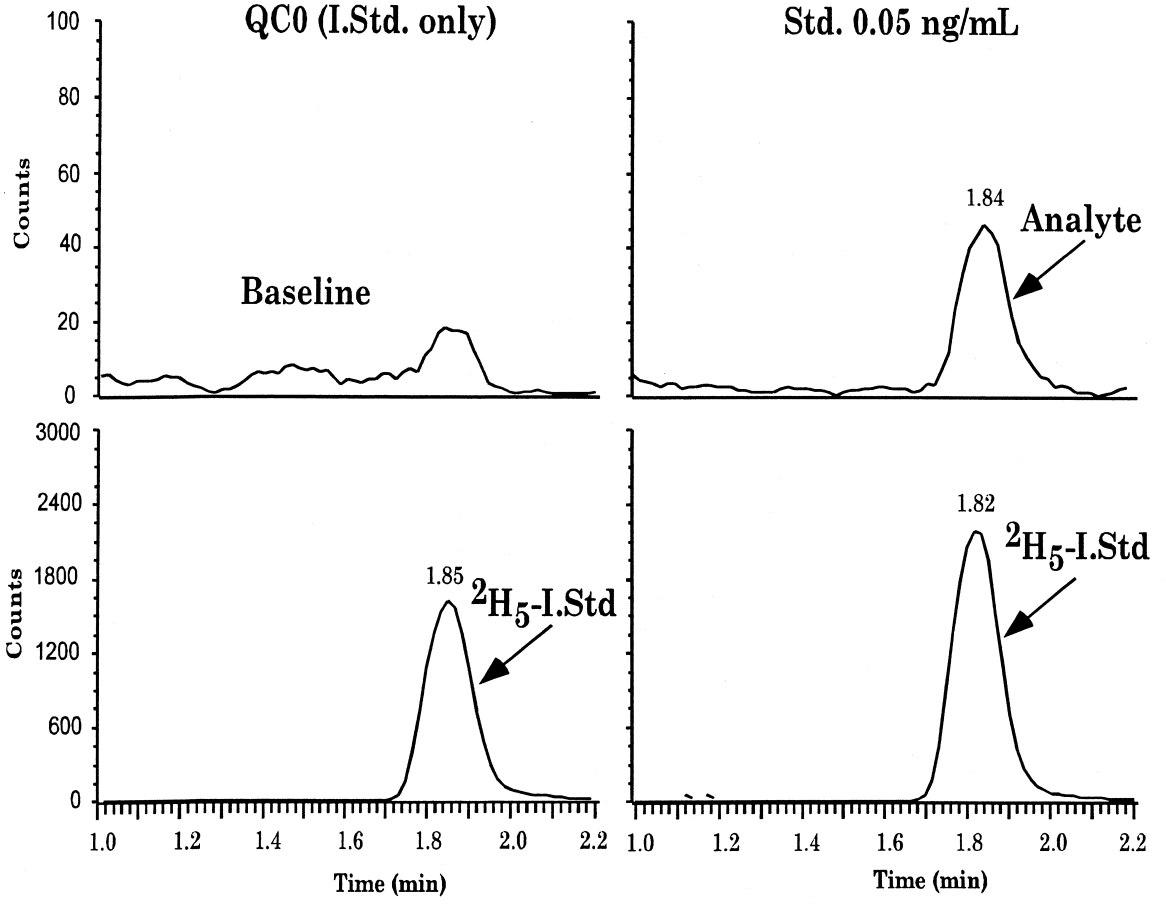

Fig. 4. SRM ion chromatograms of a plasma sample containing internal standard only at 5.0 ng/ml (QC0) and of a plasma sample containing the analyte at 50 pg/ml and the internal standard at 5.0 ng/ml (STD 0.05 ng/ml) following automated SPE. Retention time of the analyte and internal standard=1.84/1.82 min.

analyte. It should be noted that the injected amount was 40% of the reconstituted extract for both automated LLE and automated SPE but only 10% for manual LLE. The extraction recovery of the analyte from plasma was determined to be 85%, 70% and 70% for manual LLE, automated LLE and automated SPE, respectively.

4. Conclusion

Three extraction procedures, manual LLE, automated LLE and automated SPE were compared for processing plasma samples for analysis by LC–MS–MS of a carboxylic acid containing analyte. Sample preparation by automated LLE or automated SPE was approximately three-times faster than manual LLE. In addition, the time physically spent on the automated LLE or automated SPE was only a small fraction (approximately 1/25th) of the time spent on the manual LLE. The performance of the automated SPE or automated LLE, as evidenced by the accuracy and precision obtained from analysis of QC samples, is as good as that of the manual LLE method. Automation of the sample preparation significantly reduced the analyst exposure to the biological samples.

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