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Short communication

Simultaneous determination of a hydrophobic drug candidate and its metabolite in human plasma with salting-out assisted liquid/liquid extraction using a mass spectrometry friendly salt

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ABSTRACT

In previously reported applications of salting-out assisted liquid/liquid extraction (SALLE) with acetonitrile, only inorganic salts were evaluated and implemented as the salting-out reagents. A potential concern of the method for the subsequent LC–MS analysis of biological samples was that a portion of the added salt (typically of high concentration) might be extracted and affect the chromatography separation and ionization of chromatography effluents in a mass spectrometer. Here we report, for the first time, the use of a mass spectrometry friendly organic salt, ammonium acetate, as a salting-out reagent in SALLE with acetonitrile for the simultaneous quantitation of an Abbott investigational new drug ABT-869 and its hydrophilic metabolite in human plasma. The performance of SALLE with ammonium acetate was compared with that of a previously reported method with a conventional liquid/liquid extraction technique using a set of pooled incurred samples. The % differences of the measured concentrations for 24 samples from these two methods were found to be within acceptance criteria, demonstrating SALLE with ammonium acetate as a reliable sample preparation technique. The SALLE method is simple, fast (25 min/plate), easy for automation, free of drying down step, and environmentally friendly. SALLE with mass spectrometry friendly salts has been applied to regulated sample analysis of both hydrophilic and hydrophobic compounds. It is envisioned that SALLE with acetonitrile and ammonium acetate be a universal method for high throughput automated sample preparation for bioanalytical chemistry.

1. Introduction

Sample preparation is a critical part of high throughput quantitative bioanalysis. Protein precipitation (PPT), liquid/liquid extraction (LLE), and solid phase extraction (SPE) are the three most widely used sample preparation techniques [1]. LLE is simple and the extracts are clean, but the method is generally unsuitable for hydrophilic compounds. SPE works with most compounds but suffers from having a complicated procedure, relatively poor reproducibility and high cost. PPT is simple and applicable to both hydrophilic and hydrophobic compounds, but the "extract" – the supernatant – is relatively unclean since it still contains a significant amount of unprecipitated soluble plasma components that could affect chromatography separation or suppress the ionization of the target analyte.

Recently, salting-out assisted liquid/liquid extraction (SALLE) with acetonitrile has been introduced for high throughput quantitative LC–MS analysis of biological samples [2]. Although the concept of salting-out acetonitrile was reported in 1989 for bioanalysis using UV detection [3], the method has not seen application in automated sample preparation for LC–MS analysis of biological samples until our recent report.

Although SALLE with acetonitrile has demonstrated its advantages in biological sample clean-up and analyte enrichment over conventional LLE, PPT, and SPE methods, the use of magnesium sulfate, ammonium sulfate, sodium chloride, calcium chloride, potassium carbonate and calcium sulfate [2–8] can raise concerns for subsequent LC–MS analysis. In this report, we introduced a mass spectrometry friendly salt, ammonium acetate, as a new SALLE salting-out reagent. Quantitative analysis of a hydrophobic drug candidate (ABT-869) and its hydrophilic metabolite (A-849529) in human plasma using SALLE as the sample preparation method was used to evaluate the capability of this new sample preparation technique. Concentrations of ABT-869 and its metabolite from 24-pooled clinical samples were determined using both SALLE with acetonitrile and the previously reported method [9] without any

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modification. SALLE with acetonitrile and ammonium acetate was evaluated briefly with reference to US FDA Guidance for Industry-Bioanalytical Method Validation [10], 2001.

2. Experimental

2.1. Reagents

ABT-869 and A-849529 were reference standards from Abbott Laboratories (North Chicago, IL, USA). ABT-869 is a hydrophobic drug candidate and A-849529 is an active metabolite of ABT-869. A-849529 is hydrophilic. The deuterated internal standards, A-741439 D_4 and A-849529 D_4, were also from Abbott Laboratories. Water was prepared from purified de-ionized water using a Millipore Milli-Q (Billerica, MA, USA). Acetonitrile, hexanes, ethyl acetate, methanol, formic acid and glacial acetic acid were from EMD Chemicals (Gibbstown, NJ, USA). Ammonium acetate and ammonium formate was from J.T. Baker (Phillipsburg, NJ, USA). All of the above solid reagents are A.C.S. grade. Solvents such as hexanes, methanol, acetonitrile, and ethyl acetate were HPLC grade. Blank human plasma was from Biological Specialty Corporation (Colmar, PA, USA).

2.2. Instruments

An SIL-HTc autosampler and LC-10AD VP pump from Shimadzu Corporation (Kyoto, Japan) was used for the chromatography. An API-3000 mass spectrometer from MDS Sciex (Thornhill, Ontario, Canada) was used as a detector. Data was acquired and processed by Analyst 1.4.2 software, also from MDS Sciex. A laboratory information system (LIMS 7.2), from Thermo Electron Corporation (Waltham, MA, USA) was used for data storage and regression. A SymmetryShield™ column from Waters (Milford, MA, USA) and a Zorbax guard column from Agilent (Palo Alto, CA, USA) were used for the separation. A MicroLab AT Plus 2 automated liquid handler from Hamilton Company (Reno, NV, USA) was used for liquid handling. A VX2500 multi-tube vortexer from VWR (West Chester, PA, USA) was used to ensure thorough mixing. A multi-channel evaporator, modified in-house at Abbott Laboratories, was used to dry down the organic extract for the conventional liquid/liquid extraction method. A centrifuge from Jouan (West Chester, PA, USA) was used to separate the organic phase from the aqueous phase and collect the precipitated proteins in the SALLE method.

2.3. Two sample extraction procedures

Samples were prepared using a 96-well liquid/liquid extraction technique. All liquid transfers were performed by the Hamilton Microlab AT2 Plus automated liquid handler.

In the conventional liquid/liquid extraction (CLLE) with 1:11 hexanes: ethyl acetate, the extraction procedure reported in Ref. [9]. The sample preparation of a 96-well plate takes approximately 90 min.

In SALLE, 50 μL of each sample was added to the appropriate wells of a 96-well polypropylene plate. Fifty microliters of internal standard solution was then added to each well except the well for the double blank, 50 μL of 2.0 M ammonium acetate buffer was added to each well, and then 200 μL of acetonitrile was added to each well. The plate was centrifuged at 3000 rpm for approximately 3 min. One hundred microliters of the supernatant organic phase was transferred into a clean plate and then diluted by adding 100 μL of Milli-Q water into each well of the plate. The plate was capped and shaken for approximately 3 min using a multi-tube vortexer, and 20 μL of solution was serially injected into the mass spectrometer. The total sample preparation for a 96-well plate took approximately 20 min.

2.4. Preparation of calibration standards and quality controls

The calibration standards and quality control samples used to evaluate the SALLE method were previously unused solutions that had been saved for use with the previously validated conventional liquid/liquid extraction method. The preparation of calibration standards and quality control samples followed the reported method [8]. Stock solutions used for preparing standard and quality control (QC) were made from two independent weighings. Stock solutions were prepared from solid powders and dissolved with 1:1 (v/v) acetonitrile:water. Two working solutions were prepared from diluted and combined stock solutions of both ABT-869 and A-849529. Calibration standards and quality control samples were prepared by spiking designated working solutions into blank human plasma. Ten calibration standards and three quality controls were prepared in the validated range from approximately 1 ng/mL to 600 ng/mL. Aliquots of calibration standards and quality control samples were stored at approximately −70 °C until they were used.

2.5. Chromatography and tandem mass spectrometric detection

The chromatography separation followed the previously reported method [8]. A Waters SymmetryShield™ RP8, 5 μm, 2.1 mm × 150 mm analytical column with an Agilent Zorbax 300SB-C8, 5 μM, 2.1 mm × 20 mm guard column was used for separation. The flow rate was maintained at 0.3 mL/min. The mobile phase consisted of 0.1% formic acid and 50% acetonitrile in water (v/v). A solution with 0.1% formic acid and 90% acetonitrile in water (v/v) was used as a backwash solvent. The chromatography effluent was monitored using an MDS Sciex API 3000 triple quadrupole mass spectrometer with a turbo ion spray (ESI) interface. The mass spectrometer was operated in a positive ion multiple reaction monitoring (MRM) mode. The following fragmentation channels were monitored with dwell times of 200 ms: m/z 376.1 → 251.3 for ABT-869, m/z 406.1 → 251.3 for A-849529, m/z 380.2 → 255.3 for A-741439 D_4, and m/z 410.2 → 255.3 for A-849529 D_4. All other mass spectrometer parameters were optimized. Mass spectrometric data acquisition was initiated at 2.0 min and lasted for 4.4 min. Total chromatographic run time was 6.5 min.

2.6. Data processing

Peak areas of each analyte were calculated using the SCIEX Analyst™ software version 1.4.2. A calibration curve was derived from the peak area ratios versus the concentration of the standards with a weighing factor of $1/x^2$ (where x is the concentration of a given standard). The regression equation for the calibration curve was then used to back-calculate the found concentrations. For each standard and QC, the results were compared to the theoretical concentrations to obtain the accuracy, expressed as a %bias from theoretical concentration of each sample measured. Results from the QC samples were used to verify accuracy and precision of the analytical results for the study samples. Results from the pooled unknown samples were used to compare the reproducibility between the SALLE and conventional liquid/liquid extraction methods.

3. Results and discussion

3.1. SALLE with ammonium acetate

There have been limited reports on SALLE with water miscible organic solvents and inorganic salts as the salting-out agent, for the separation, isolation and preparation of biological samples. These inorganic salts included ammonium sulfate, sodium chloride, potassium carbonate and magnesium sulfate. Rustum [3] reported a salting-out approach for diltiazem analysis using anhydrous K_2CO_3 solid to separate the organic phase from the aqueous phase after protein precipitation of serum/whole blood samples with acetonitrile in 1989. Li and Huie [4] used a similar approach to prepare urine samples for capillary electrophoresis analysis of porphorins with 5 M NaCl + 100 mM phosphate as salting-out reagent following protein precipitation of urine samples in 2006. Yoshida et al. [5] published a method using 0.2 g NaCl solid added to 0.5 mL plasma for phase separation, with final NaCl concentration of 6.8 M used for GC–MS and LC–MS applications in 2004. Salting-out with inorganic salt NaCl was also used in the extraction of γ-hydroxybutyrate (GHB) and its precursors with t-butylmethylether extraction. Alternatively, phase separation without salt was also achieved at sub-zero temperature without using salt, but the method is more difficult to operate and the throughput is limited.

In these bioanalytical applications, solid inorganic salts (K_2CO_3 and NaCl) were used in sample preparations. The use of solid salts is time-consuming, difficult to automate, and raised concerns of fouling HPLC columns/mass spectrometers. In Yoshida's work, the author clearly expressed that concern by the with the following statement: "The acetonitrile phase separated by the salting-out method might contain salts as well as water, which would be harmful to mass spectrometers. For identification of drug by GC–MS or LC–MS, drugs should be extracted by the subzero-temperature extraction rather than the salting-out method". Such concerns might be the key reason that the salting-out approach has not gained much application in quantitative bioanalysis using LC–MS.

In this communication, we proposed and evaluated a universal method, SALLE, by using mass spectrometry friendly salts such as ammonium acetate and ammonium formate as the salting-out reagents. Although a small portion of the salt may dissolve into the acetonitrile phase, the impact of the salts on the subsequent LC–MS analysis will be insignificant since the salts are compatible with both liquid chromatography and mass spectrometry. In fact, the presence of ammonium acetate minimizes the formation of Na^+ adduct and thus enhances the MS response. Additionally, the approach can be easily implemented for automation, simply by adding a concentrated ammonium acetate solution instead of salt. Thirdly, we found out that there is no need to add very high concentration of salts to achieve the best separation, in contrast to what was done in the cases of Rustum and Yoshida. In fact, salt concentration in final plasma samples at 1 M is sufficient to produce clear and clean phase separations, and the use of high concentration of salts may produce high CV in subsequent LC–MS analysis.

SALLE has a number of advantages over those well-established methods such as protein precipitation, liquid/liquid extraction, SPE, and online SPE. Since SALLE is essentially an LLE extraction, analytes can be concentrated when needed, simply by adjusting the relative volume of sample to acetonitrile. Typically, the extract can be diluted by water and then injected for LC–MS analysis directly, without the drying down step as typically required for other separation techniques. This especially true for non-clinical assays which typically have a relatively high LLOQ (greater than 1 ng/mL). Compared with PPT, it is a cleaner extract without a drying down and reconstitution. Compared with LLE, which typically requires drying down to remove organic solvents and reconstitution for subsequent analysis, it is a faster and more environmentally friendly plate preparation. When compared with offline SPE, SALLE is much simpler and faster in operation. Compared with online SPE such as turbulent flow chromatography, which typically operates at very high flow rate, SALLE uses much less solvent, can be used for analysis requiring low LLOQs, and does not require additional pumps.

SALLE is a more green technique compared with common sample preparation techniques used for bioanalysis. For non-clinical and clinical studies requiring relatively high LLOQs, no drying-down step is required compared with PPT, SPE and offline SPE. Therefore, SALLE emites significantly less toxic solvent into the air. It also uses much less solvent compared with online sample preparation techniques which require daily preparation of 5–10 L of solvents per instrument.

3.2. Comparison of LLE with hexanes:ethyl acetate and SALLE with acetonitrile

3.2.1. Accuracy and precision comparison of LLE and SALLE

One batch was run for the accuracy and precision evaluation of SALLE with acetonitrile. This batch included three replicates of quality control samples. Both LLE with hexanes: ethyl acetate and SALLE with acetonitrile show similar accuracy and precision. The Standard and QC performance results are listed in Table 1.

3.2.2. Extraction recovery

In order to measure extraction recovery, three recovery controls (RC) were prepared in neat solution and three-recovery evaluation (RE) QCs were prepared in human plasma. Three concentrations of RCs are the same as the concentrations of RE QCs. Fifty microliters

Table 1
Performance of standards and QCs

	ABT-869			A-849529		
	Concentration (ng/mL)		%Bias	Concentration (ng/mL)		%Bias
STD 1	**1.20**	1.21	0.98	**1.21**	1.21	0.06
STD 2	**5.98**	5.75	−3.8	**6.05**	6.08	0.59
STD 3	**12.0**	11.7	−1.8	**12.1**	12.2	0.59
STD 4	**17.9**	17.8	−0.92	**18.1**	17.9	−1.6
STD 5	**23.9**	23.9	−0.21	**24.2**	23.7	−2.2
STD 6	**59.8**	59.9	0.21	**60.5**	59.8	−1.2
STD 7	**120**	122	2.5	**121**	120	−0.90
STD 8	**359**	364	1.6	**363**	374	3.0
STD 9	**478**	481	0.60	**484**	492	1.8
STD 10	**574**	579	0.95	**581**	580	−0.16
R^2			0.9999			0.9997

Note: Concentrations in bold are theoretical concentrations.

Table 2
Extraction recovery across different salts and concentrations (%)

Salt solution	ABT-869			A-849529		
	QC low	QC mid	QC high	QC low	QC mid	QC high
2 M ammonium acetate	35.3	34.5	37.7	19.7	18.0	20.1
10 M ammonium acetate	34.7	34.2	36.9	19.4	18.1	20.3
2 M ammonium formate	33.3	34.1	36.5	17.9	18.2	20.4

Table 3
A summary of matrix effect evaluation samples

ABT-869			A-849529		
Sample ID	Found Conc. (ng/mL)	%Bias	Sample ID	Found Conc. (ng/mL)	%Bias
ME-A	3.90	−0.93	ME-A	3.97	2.58
ME-B	3.84	−2.64	ME-B	3.74	−3.30
ME-C	3.78	−3.98	ME-C	3.69	−4.69
ME-D	3.94	0.01	ME-D	3.94	1.83
ME-E	3.98	1.09	ME-E	3.94	1.87
ME-F	4.01	1.73	ME-F	4.13	6.80
ME-Control	**3.94**		ME-Control	**3.87**	

Note: Concentrations in bold are reference concentrations.

of RE QC sample was extracted as a normal sample and injected. The recovery controls were prepared as follows: 50 μL of blank human plasma was extracted as normal samples, 100 μL of extract was transferred into the injection plate and 50 μL of the appropriate RC solution and 50 μL of Milli-Q water was added into the wells. The plate was injected as usual and the RE/RC ratios of the peak area ratios were calculated to determine extraction recovery. Two different strengths of ammonium acetate solution were evaluated (2 M and 10 M) as well as a 2 M solution of ammonium formate. The extraction recovery from SALLE with acetonitrile is adequate for accurate and precise results with all concentration levels and for both salts. This result helps justify the use of only half the sample volume of the originally published method. The extraction recovery results are listed in Table 2. Achieved extraction recoveries from SALLE are comparable to the data from LLE reported in Ref. [9]. The extraction recovery was based on approximately 50% of the acetonitrile phase transfer from total 200 μL of acetonitrile phase formed in the upper layer.

3.2.3. Matrix effect

In order to prove that the SALLE method is independent of individual matrix lot variation, seven matrix effect QCs were prepared. One control matrix effect QC was prepared in the plasma lot that was used for preparing calibration standards. Six other evaluation matrix effect QCs were prepared in six additional lots of plasma. All seven matrix effect QCs were prepared at a concentration similar to that of the low concentration quality control sample and were extracted as unknown samples. The mean found concentration of the control matrix effect QC was used as the comparator for evaluating matrix effect QCs. Calculated mean %bias was between −4.0% and 1.7%. There was no matrix effect difference observed from SALLE with acetonitrile. The matrix effect results can be found in Table 3.

3.2.4. Selectivity

In order to prove that the SALLE method is free from interference from endogenous matrix components, six lots of blank matrix were tested. These blank matrix samples were extracted with internal standard. None of the extracted lots of plasma showed peaks with areas above 5% of the peak area for either ABT-869 or A-849529.

Table 4
Peak area of selectivity samples

ABT-869			A-849529		
Sample ID	Peak area	% of LLOQ	Sample ID	Peak area	% of LLOQ
Blank A	0	0	Blank A	0	0
Blank B	0	0	Blank B	0	0
Blank C	0	0	Blank C	258	4.8
Blank D	0	0	Blank D	0	0
Blank E	0	0	Blank E	0	0
Blank F	0	0	Blank F	0	0
LLOQ	16050		LLOQ	5335	

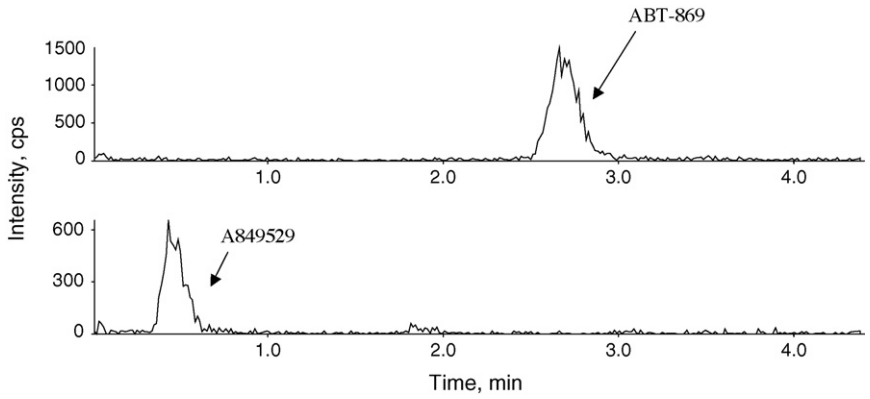

Fig. 1. A chromatogram of LLOQ sample at approximately 1 ng/mL each.

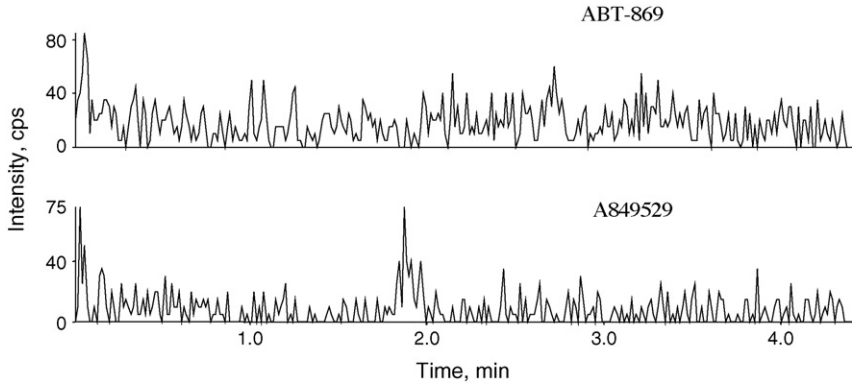

Fig. 2. A chromatogram of blank samples from SALLE with acetonitrile.

Table 5
Cross-validation results with two extraction methods

Plasma pool	Time (h)	ABT-869			A-849529		
		LLE	SALLE	%Difference	LLE	SALLE	%Difference
A	0	111	101	−9.0	120	121	0.8
A	0.5	146	141	−3.4	73.1	73.2	0.1
A	1	316	308	−2.5	138	140	1.4
A	2	367	362	−1.4	108	112	3.7
A	3	350	344	−1.7	183	186	1.6
A	4	331	326	−1.5	136	134	−1.5
A	6	281	274	−2.5	149	149	0.0
A	8	250	243	−2.8	159	156	−1.9
B	0	93.3	86.8	−7.0	86.0	86.9	1.0
B	0.5	114	110	−3.5	84.1	82.9	−1.4
B	1	179	173	−3.4	82.7	82.0	−0.8
B	2	267	266	−0.4	95.1	95.9	0.8
B	3	306	303	−1.0	107	105	−1.9
B	4	283	270	−4.6	123	121	−1.6
B	6	188	182	−3.2	74.4	75.1	0.9
B	8	200	190	−5.0	112	112	0.0
C	0	24.9	20.2	−18.9	50.2	49.8	−0.8
C	0.5	48.9	45.2	−7.6	48.2	49.4	2.5
C	1	106	102	−3.8	51.2	53.3	4.1
C	2	129	119	−7.8	54.7	56.4	3.1
C	3	141	135	−4.3	71.1	73.0	2.7
C	4	138	127	−8.0	88.6	92.1	4.0
C	6	108	99.4	−8.0	95.7	97.7	2.1
C	8	90.1	81.5	−9.5	86.2	87.1	1.0

Table 4 shows results for selectivity. Fig. 1 presents a chromatogram for an LLOQ sample, while Fig. 2 shows a chromatogram of blank matrix with internal standard added.

3.2.5. Cross-validation of two methods with incurred samples

From one dose group of a clinical study, multiple non-placebo samples collected from different subjects at the same time were pooled into 24 unknown samples. The pooled samples went through two independent extractions and analyzed against 10 calibration standards. Three replicates of quality control samples were also included in each batch. The calculated concentrations from the previously validated LLE method were used as the control values. The found concentrations are listed in Table 5. For ABT-869, 23/24 samples extracted with the SALLE method were found to be within ±15% of the control values. The % difference was between −18.9% and −0.4%. For A-849529, 24/24 samples extracted with the SALLE method were found to be within ±15% of the control values. The % difference was between −1.9% and 4.1%. Measured concentrations of ABT-869 and A-849529 from different extraction methods proved that SALLE with acetonitrile was adequate for the accurate determination of ABT-869 and A-849529 in human plasma.

4. Conclusion

In summary, ammonium acetate has been used in SALLE with acetonitrile as a simple and fast sample preparation technique for quantitative LC–MS/MS analysis. Due to the volatility and compatibility of acetonitrile with reversed phase chromatography, post salting-out extract was injected directly into LC–MS/MS system after simple dilution. This approach has been successfully used for simultaneous determination of a hydrophobic drug candidate and its metabolite. Comparison of quantification results of clinical samples using this approach and a conventional LLE demonstrated that SALLE using acetonitrile and ammonium acetate is suitable for GLP/GCP analysis of biological samples. To date, dozens of GLP methods for more than 12 drug candidates in urine and plasma have been validated using this approach. It is envisioned that this approach will become a universal sample preparation approach in bioanalytical chemistry.

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