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Note

Liquid chromatographic determination of low-molecular-weight amides in pharmaceutical matrices

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Low-molecular-weight amides, such as N,N-dimethylformamide (DMF) and N,N-dimethylacetamide (DMAC), are common solvents in the chemical, agricultural, and pharmaceutical industries. Because of the potentially toxic properties of amides it is necessary to control their concentrations at very low levels in isolated products[1]. This requires a sensitive, accurate, and reproducible analytical method for the separation and quantitation of these analytes in organic matrices.

Gas chromatography (GC) has commonly been used for the separation and quantitation of low-molecular-weight amides in air or aqueous matrices[2,3]. GC methods for more complex matrices suffer from low sensitivity and interferences from the matrix. High-performance liquid chromatography (HPLC) was investigated as an alternative to GC for the determination of amides in several pharmaceutical compounds. In this note, we report methods for the separation and quantitation of acetamide, DMF and DMAC in complex matrices by HPLC. As examples, we present three applications of the method which have been studied in detail, including comparisons of HPLC and GC methods.

EXPERIMENTAL

Materials

Acetonitrile (HPLC grade) was purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.). All other chemicals employed were of reagent grade and were used without further purification. The water used was deionized and passed through a Millipore (Bedford, MA, U.S.A.) Milli-Q water purification system. All samples were powders of the bulk material obtained from Eli Lilly (Lafayette, IN, U.S.A.).

Chromatographic systems

HPLC experiments were conducted using a Spectra-Physics 8700 solvent-delivery system (San Jose, CA, U.S.A.) with a Micromeritics 728 autosampler (Norcross, GA, U.S.A.), a Valco fixed-loop injection valve (Houston, TX, U.S.A.) and a Kratos 757 variable-wavelength UV detector (Ramsey, NJ, U.S.A.). The chromatographic conditions were as follows: column, 250 mm × 4.6 mm I.D. Zorbax C_8 (DuPont, Wilmington, DE, U.S.A.) or Alltech C_{18} (Deerfield, IL, U.S.A.); mobile phase, 3–5% acetonitrile in 0.1 *M* phosphate buffer; flow-rate, 1–1.5 ml/min; temperature, ambi-

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ent; injection volume, 10–50 μl. All samples and standards were dissolved in 0.1 *M* phosphate buffer.

GC experiments were conducted using a Hewlett-Packard 5880 gas chromatograph with a flame ionization detector (Palo Alto, CA, U.S.A.) or a Tracor 570 gas chromatograph equipped with a nitrogen–phosphorus detector (Austin, TX, U.S.A.). The data were collected and processed with a Hewlett-Packard 1000 computer and analytical software developed in-house. The GC columns were obtained from the following sources: DB columns, J&W Scientific (Folsom, CA, U.S.A.); Porapak, Chromosorb, and OV-101 columns, Supelco (Bellefonte, PA, U.S.A.); Tenax, Scientific Products (McGaw Park, IL, U.S.A.).

RESULTS AND DISCUSSION

Determination of amides by HPLC

In the analysis of pharmaceutical samples for low-molecular-weight amides, separation of the analyte from the sample matrix is an important factor in obtaining adequate sensitivity. In this study, a reversed-phase HPLC system was developed which strongly retained the sample matrix while allowing the analyte to be eluted and detected. The baseline remained stable and free from interferences from the sample matrix for *ca.* ten injections before matrix components began to be eluted from the column. When these interferences were observed, the sample matrix was removed from the column by increasing the strength of the mobile phase. Although more time-consuming, the method could be automated by utilizing gradient elution after each injection as described in the section on the determination of DMAC. A wavelength of 210 nm was necessary for the detection of amides. As shown in Fig. 1, seven different amides were resolved under the conditions given. When this method was applied to different products, only slight modifications were necessary. The strength of the mobile phase, pH and flow-rate were adjusted to resolve the peak from the

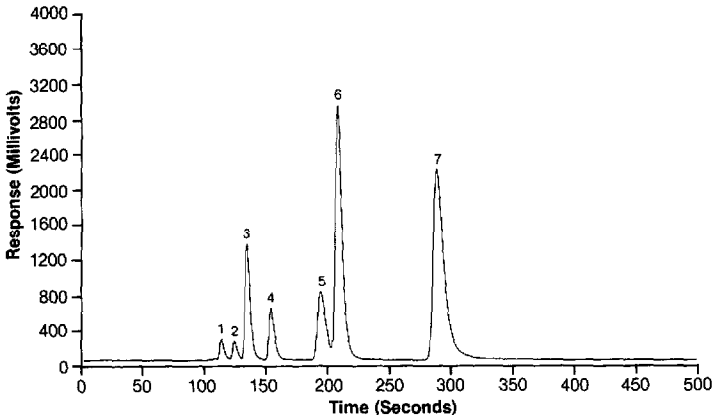

Fig. 1. HPLC chromatogram of low-molecular-weight amides. Column, Zorbax C_8 (5 μm, 250 mm × 4.6 mm I.D.); eluent, acetonitrile–0.1 *M* phosphate buffer, pH 2.5 (5:95); flow-rate, 1.5 ml/min; detection, 210 nm; temperature, ambient; injection volume, 10 μl. Peaks: 1 = formamide; 2 = acetamide; 3 = N-methylformamide; 4 = N-methylacetamide; 5 = N-ethylformamide; 6 = N,N-dimethylformamide; 7 = N,N-dimethylacetamide; all at a concentration of 0.1 mg/ml.

sample matrix, impurities, and baseline disturbances at the void volume. When increased sensitivity was required, the injection volume was increased.

Determination of acetamide

Previous methods for the determination of acetamide by GC utilized Tenax or a porous polymer, such as Poropak or Chromosorb, as the column packing material[4]. However, it was noted that with porous polymer packings quantitation of polar compounds, such as acetamide, was difficult. Below 100 μg/g, non-linear behavior and adsorption of the compounds by the column packing material were observed[5].

A Tenax column was evaluated for the GC determination of acetamide in a pharmaceutical product. The peak shape was acceptable but acetamide gave a low response in the flame ionization detector. A linearity and matrix study was performed by preparing a set of standards and spiked samples, covering a range from 10 to 200% of the target level in the sample. For example, if the maximum target level for the amide was 100 μg/g, standards and spiked samples were prepared covering a range of 10 to 200 μg/g of amide in the sample. Log–log slopes and coefficients of determination (r^2) were calculated from the least-squares line of the response–concentration plot for both standards and spiked samples. A linear response was indicated when the log–log slope of the least-squares line did not differ significantly from 1.0 and the coefficient of determination approached 1.0. To determine whether there was a matrix effect, the least-squares slope of the spiked samples was compared to that of the standards. Slope ratios differing significantly from 1.0 indicate the presence of a matrix effect[6,7]. The linearity and matrix validation are summarized in Table I. In this example, the slope ratio of 0.94 corresponded to a 6% error in the determination of the concentration over the concentration range tested. The negative intercept and the depressed log–log slope observed could be indicative of adsorption at low levels. Based on the sample concentration of 100 mg/ml, the detection limit was 50 μg/g for the GC–flame ionization detection of acetamide. In an effort to improve the sensitivity and eliminate the matrix effects, a nitrogen–phosphorus detector was evaluated. No improvement in sensitivity was observed. For this application, a detection limit of 10 μg/g or less, based on the sample weight was required.

Attempts to increase the sensitivity by increasing the sample concentration were not successful. At sample concentrations greater than 100 mg/ml, interferences from

TABLE I

SUMMARY OF VALIDATION STATISTICS

Analyte	*Method*	*Concentration range (μg/g)*	*Sample set*	*Slope*	*y intercept*	r^2	*log–log slope*	*Slope ratio*
Acetamide	GC	100–2000	Standards	$5.3 \cdot 10^4$	$-6.8 \cdot 10^2$	0.9964	0.84	
			Spikes	$5.0 \cdot 10^4$	$-7.6 \cdot 10^2$	0.9857	0.81	0.94
Acetamide	HPLC	10–200	Standards	9.1	$-2.4 \cdot 10^1$	0.9998	1.00	
			Spikes	8.2	$-1.5 \cdot 10^1$	0.9986	1.11	0.90
DMAC	HPLC	60–1300	Standards	$2.3 \cdot 10^6$	$-9.7 \cdot 10^1$	0.9993	1.01	
			Spikes	$2.3 \cdot 10^6$	$5.0 \cdot 10^4$	0.9985	1.05	1.01
DMF	HPLC	1–55	Standards	$8.1 \cdot 10^2$	$1.4 \cdot 10^2$	1.0000	1.00	
			Spikes	$8.0 \cdot 10^2$	$1.5 \cdot 10^2$	1.0000	1.02	0.98

TABLE II

GC CONDITIONS EVALUATED FOR THE DETERMINATION OF ACETAMIDE IN A PHARMACEUTICAL MATRIX

Solvent	*Column*	*Temperature (°C)*	*Comments*
Water	30 m × 0.32 mm I.D., 0.25-μm film DB-1	100	Detection limit 100 μg/g
Water	60 m × 0.32 mm I.D., 1-μm film DB-1	40–200	Detection limit 250 μg/g
Water	30 m × 0.53 mm I.D., 1.5-μm film DB-1	100	Irreproducible peaks
Methanol	30 m × 0.53 mm I.D., 1.5-μm film DB-1	40–250	Poor peak shape
Water	30 m × 0.53 mm I.D., 5-μm film DB-1	40–100	Poor peak shape
Water	15 m × 0.53 mm I.D., 1-μm film DB-17	40	Poor peak shape
Water	15 m × 0.53 mm I.D., 1-μm film DB-Wax	40	Poor peak shape
Methanol	15 m × 0.53 mm I.D., 1.5-μm film DB-1	40–80	Poor peak shape
Water	15 m × 0.53 mm I.D., 1.5-μm film DB-1	40–80	Irreproducible peaks
DMF	15 m × 0.53 mm I.D., 1.5-μm film DB-1	40–80	Coelutes with solvent
Water	6 ft. × 2 mm I.D., Porapak Q	100–250	Not eluted
Water	6 ft. × 2 mm I.D., Chromosorb 104	200–240	Sample builds up on column
Methanol	6 ft. × 2 mm I.D., 3% OV-101	40–200	Coelutes with solvent
DMF	6 ft. × 2 mm I.D., 3% OV-101	40–200	Coelutes with solvent
Water	6 ft. × 2 mm I.D., 3% OV-101	40–200	Irreproducible peaks

the sample matrix were severe. This was mainly due to the buildup of non-volatilized sample on the column and decomposition of the sample in the injector. As shown in Table II, other attempts to increase the sensitivity included: alternate column packings, narrow- and wide-bore capillary columns, and different sample solvents.

The HPLC method was explored as an alternative to GC. Conditions were developed for the determination of acetamide, and a linearity and matrix study was conducted covering the range from 10 μg/g to 200 μg/g, based on the sample concentration of 100 mg/ml. An example of the resulting HPLC elution profile is shown in

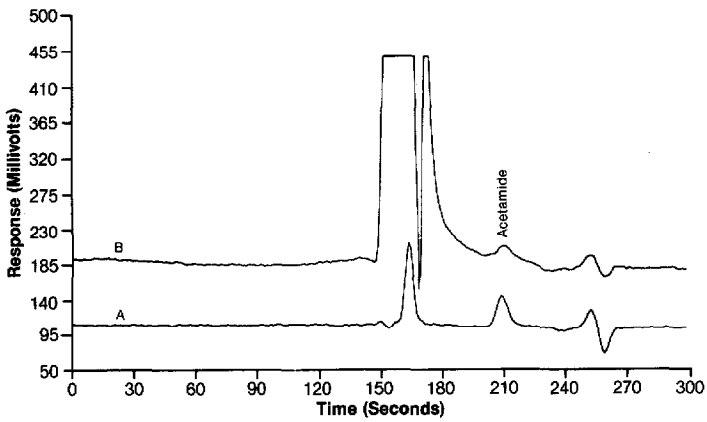

Fig. 2. HPLC chromatograms of acetamide in a pharmaceutical product. Column, Alltech C_{18} (10 μm, 250 mm × 4.6 mm I.D.); eluent, acetonitrile–0.1 *M* phosphate buffer, pH 4.4 (3:97); flow-rate, 1.0 ml/min; detection, 210 nm; temperature, ambient; injection volume, 10 μl. Samples: (A) standard acetamide solution (1 μg/ml); (B) sample (100 mg/ml) in phosphate buffer. Peak corresponds to a spike of 10 μg/g acetamide.

Fig. 2. The validation summary given in Table I indicates a matrix effect by HPLC, comparable to that observed by GC. However, a significant improvement in the detection limit by HPLC was observed. The detection limit by HPLC for acetamide in the pharmaceutical product was statistically determined to be 10 $\mu g/g$[8].

Determination of DMAC

Another application of the HPLC method for amides was the determination of DMAC in an antibiotic pharmaceutical product. GC has been the traditional method of choice for determining DMAC[9]. GC determinations were attempted with both packed columns containing Chromosorb 102 and DB-1 wide-bore capillary columns. Good resolution was obtained on both types of column. Unfortunately, the non-volatile product matrix built up at the front of the column and led to gradually increasing baselines and analyte carryover from one injection to the next. DMAC was observed in solvent blanks injected after sample injections. Fig. 3 is an illustration of the effect of sample injection on the baseline response and analyte carryover. Scan B shows a positive response for a sample which, because of the synthesis used, contained no DMAC. Scan C shows a spiked sample, which indicated a recovery of 140%. On a new DB-1 column, a detection limit of 0.2 mg/g was obtained. However, the sensitivity gradually decreased as samples were injected into the column. A level of detection of *ca.* 0.1 mg/g DMAC in the sample matrix was required.

The HPLC assay was developed and tested for linearity and matrix effects. Validation covered a concentration range of 0.06 to 1.3 mg/g, based on a sample concentration of 10 mg/ml. As shown in Table I, both spiked and unspiked calibration curves were linear with adequate coefficients of determination. A matrix effect was not observed in this application. The reproducibility of the method was estimated from results for control samples generated over a 6-month period. The assay was performed on ten separate days by two different analysts. The same instrument and column were used in each case. The resulting mean was 0.46 mg/g with a standard

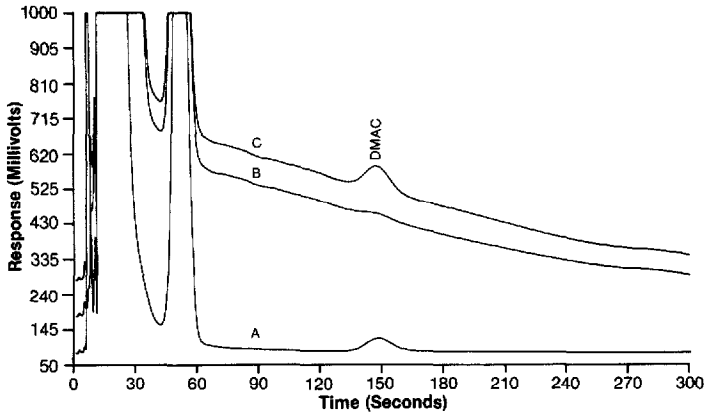

Fig. 3. GC chromatograms of DMAC in an antibiotic matrix. Column, Chromosorb 102 (3 ft. × 2 mm I.D., 80–100 mesh); carrier gas, helium; flow-rate, 25 ml/min; flame ionization detection; temperature, 200°C; injection volume, 12 μl. Samples: (A) standard DMAC solution (10 $\mu g/ml$); (B) sample (10 mg/ml) in dichloromethane; (C) sample spiked with the 10 $\mu g/ml$ DMAC standard.

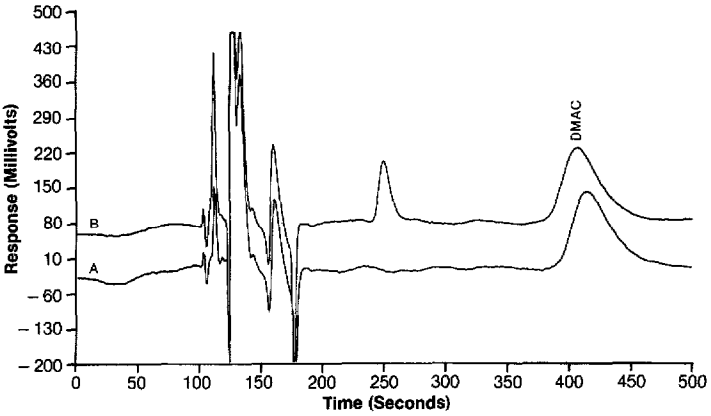

Fig. 4. HPLC chromatograms of DMAC in an antibiotic product. Column, Zorbax C_8 (5 μm, 250 mm × 4.6 mm I.D.); eluent, acetonitrile–0.1 *M* phosphate buffer, pH 2.5 (5:95); flow-rate, 1.5 ml/min; detection, 210 nm; temperature, ambient; injection volume, 10 μl. Samples: (A) standard DMAC solution (3 μg/ml); (B) sample (10 mg/ml) in methanol. Peak corresponds to a level of *ca.* 0.3 mg/g DMAC.

deviation of 0.05 mg/g. Chromatograms of standard and sample solutions are shown in Fig. 4. After *ca.* ten sample injections, gradient elution was used to remove matrix components. Acetonitrile in the mobile phase was increased from 5% to 50% over 5 min. After holding at 50% for 5 min, the acetonitrile was reduced to 5% over 5 min. The system was allowed to reequilibrate for 5 min prior to the next injection. The detection limit for DMAC in the product was *ca.* 0.05 mg/g in the HPLC method.

Determination of DMF

Residual DMF has also been determined in bulk pharmaceuticals by GC[9,10]. Following the successful use of HPLC in the previous two applications, the method was also evaluated for the determination of DMF in an agricultural product. There

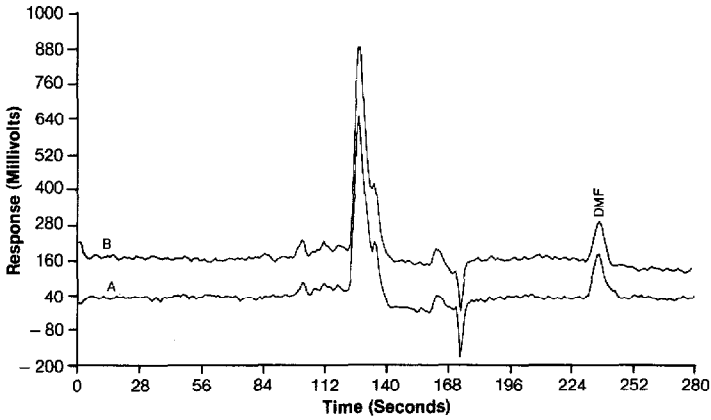

Fig. 5. HPLC chromatograms of DMF in an agricultural product. Column, Zorbax C_8 (5 μm, 250 mm × 4.6 mm I.D.); eluent, acetonitrile–0.1 *M* phosphate buffer, pH 2.5 (5:95); flow-rate, 1.5 ml/min; detection, 210 nm; temperature, ambient; injection volume, 50 μl. Samples: (A) standard DMF solution (0.05 μg/ml); (B) sample (50 mg/ml) in methanol. Peak corresponds to a spike of 1 μg/g DMF.

were no attempts to develop a GC method. The HPLC method was evaluated in terms of linearity, matrix effects, and sensitivity. The detection limit for the assay was *ca.* 0.2 μg/g DMF in the sample matrix, which relates to a total of 0.5 ng of DMF on the column. This increased sensitivity was achieved by injecting 50 μl of the sample solution as opposed to 10-μl injections in the previous two applications. In addition, a larger relative response was observed for DMF as compared to the other amides tested (Fig. 1). Assay validation results are given in Table I. Chromatograms of standard and spiked sample solutions are shown in Fig. 5.

CONCLUSION

The use of HPLC with UV detection has been described as an effective method for the determination of low-molecular-weight amides in organic matrices, such as pharmaceutical and agricultural products. By strongly retaining the sample matrix and allowing the amide analyte to elute, the method can be generally applied to many types of organic matrix. This method was found to exhibit greater sensitivity and freedom from matrix interferences than GC in the examples described.

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